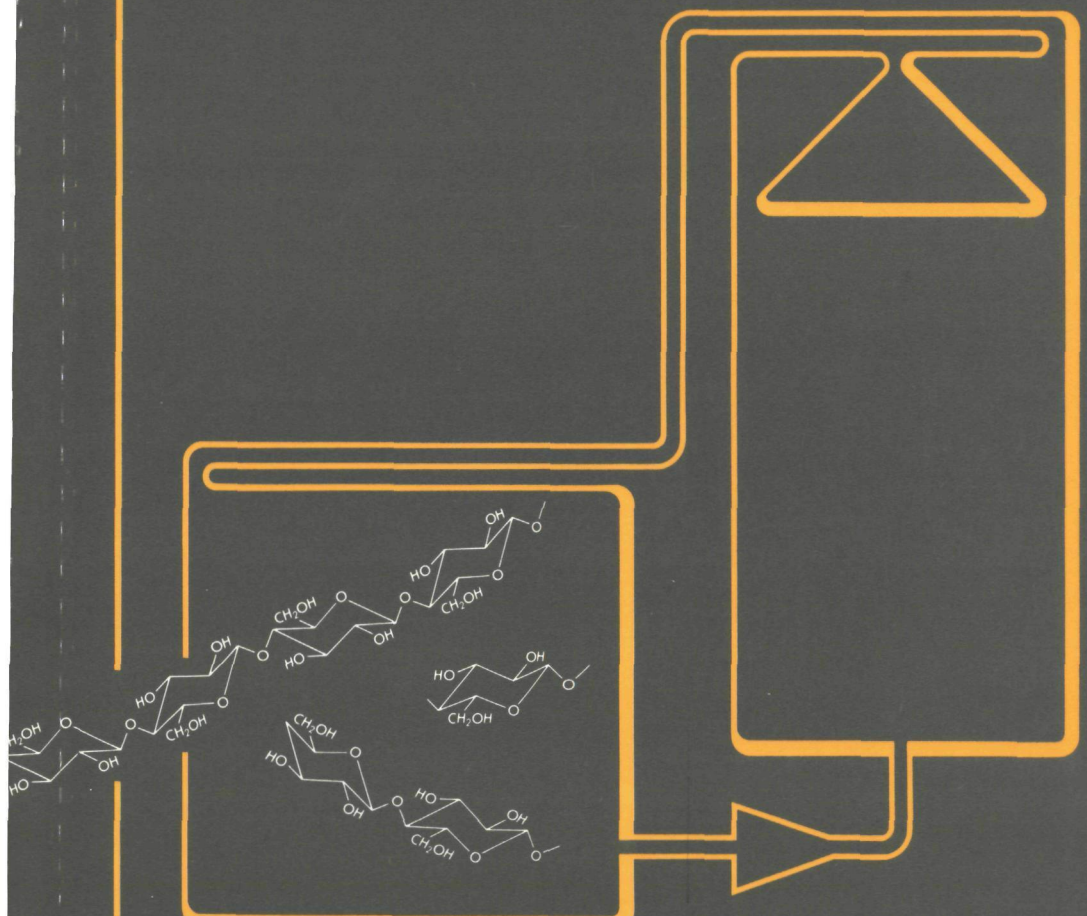
# ANAEROBIC DIGESTION OF CELLULOSIC WASTE BY A RUMEN-DERIVED PROCESS



HUUB GIJZEN

# ANAEROBIC DIGESTION OF CELLULOSIC WASTE BY A RUMEN-DERIVED PROCESS

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Gijzen, Hubertus Johannes

Anaerobic digestion of cellulosic waste by a rumen-derived process / Hubertus Johannes Gijzen. - [S.l. : s.n.] (Meppel : Krips Repro). - Ill.
Thesis Nijmegen
ISBN 90-9001563-9
SISO 579 UDC 579.6(043.3)
Subject headings: microbiology / anaerobic digestion / cellulosic waste.

# ANAEROBIC DIGESTION OF CELLULOSIC WASTE BY A RUMEN-DERIVED PROCESS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. B.M.F. VAN IERSEL VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 26 MAART 1987 DES NAMIDDAGS TE 3.30 UUR

DOOR

HUBERTUS JOHANNES GIJZEN

GEBOREN TE KERKRADE

Promotor : Prof. Dr. Ir. G.D. Vogels
Co-referent : Dr. K.B. Zwart

The investigations described in this thesis were carried out at the Department of Microbiology, University of Nijmegen, The Netherlands, and were supported by the Technology Foundation (STW).

# CONTENTS

CHAPTER 1

# GENERAL INTRODUCTION

The cyclic conversion of carbon by means of photosynthesis and microbial degradation is of utmost importance for all life on earth. The gain of this consecutive reduction and oxidation of carbon is the capture of solar energy which is used for growth and maintenance of living cells. Worldwide photosynthetic fixation of $CO_2$ is estimated to yield annually up to $1.5\text{-}2.10^{11}$ tons of dry plant material[11,55,126,145] During the process of $CO_2$ reduction by solar energy, cellulose (28-50%), hemicellulose (20-30%) and lignin (18-30%) are produced as main constituents of plant biomass[133]. Minor constituents of plant material are pectin, starch, lipids, and protein.

The decomposition of cellulosic biomass is carried out almost exclusively by microbial oxidations under both aerobic and anaerobic conditions[46,89,129]. Most of the biomass in natural environments is oxidized by aerobic microorganisms. However, a substantial amount is

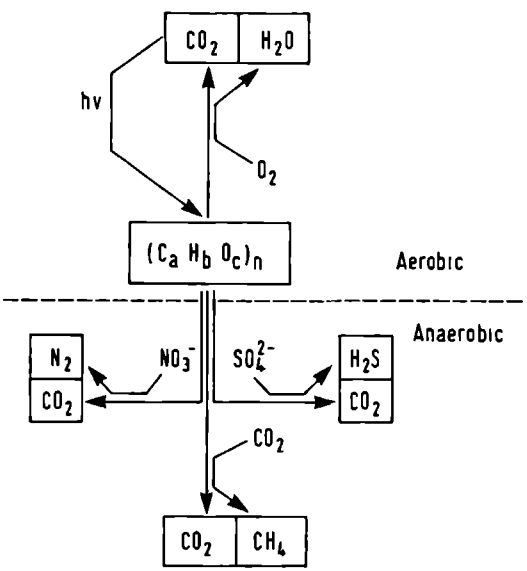



Figure 1. Main routes of decomposition of organic material in the presence of different electron-acceptors

mineralized in anaerobic environments. In this case the organic substrate is oxidized with inorganic electron acceptors such as $NO_3^-$ (nitrate reduction), $SO_4^{2-}$ (sulfate reduction) or $CO_2$ (methanogensis) (Fig 1)[31,111,112]. Anaerobic degradation to methane accounts for about 5-10% of the overall mineralization of organic matter[43,142]. Methane production occurs in anaerobic habitats such as marshes, aquatic sediments, sewage sludge digesters, paddy-fields, and the digestive tract of many herbivorous animals. Methane production by termites was recently estimated to be 40% of the global production[158] although others[117] reported a contribution of only 2-15%. The rumen is probably the most investigated methane-producing ecosystem and has been subject of many reviews[39,59,68,69,71,116,151]. Methanogenesis does not occur in environments where oxygen, sulfate or nitrate are readily available as electron acceptors[24].

## ENERGY FROM WASTE MATERIALS

A part of the photosynthetic biomass ends up as waste residue from agricultural, industrial and domestic processes, resulting in a deterioration of the environment. Because of the increasing amount of cellulosic wastes and a lack of dumping sites, the costs of current disposal methods such as dumping or incineration are increasing rapidly. On the other hand cellulosic residues represent an abundant and inexpensive renewable resource for the generation of energy and usefull products. However, the use of this potential resource requires the conversion of the biomass into a suitable form such as gaseous ($CH_4$) or liquid (ethanol, methanol) products. In view of the worldwide need for renewable energy sources, present research is focused on the utilization of biomass as an alternative to petroleum for the production of fuel and chemicals[83,88,124,135]. A variety of methods are being explored which range from physical treatments, such as pyrolysis, to chemical and biological methods such as acid or enzymic hydrolysis and fermentation. The valorization of cellulosic residues by means of physical, chemical or biotechnological methods is dependent upon the development of an economically feasible process.

Because of the relatively high moisture content of most biomass, and the high treatment costs of physical and chemical hydrolysis, a biotechnological process is more attractive.

Anaerobic digestion of organic waste may be considered as a rather simple technology for the solution of two concurrent problems: the disposal of solid waste generated by society and the need for new sources of energy. In contrast to other processes iamed at the valorization of organic waste, (e.g. methanol, ethanol production), the gaseous end products of methanogenic degradation are spontaneously separated from the liquid saving the need for an additional expensive separation step (distillation, extraction).

## ANAEROBIC DIGESTION

Anaerobic decomposition of complex organic biopolymers to methane is brought about by the combined action of a wide range of organisms. According to the present knowledge, overall anaerobic digestion is considered to proceed according to the reaction scheme depicted in Fig 2. The degradation pattern in Fig 2 is necessarily simplified, especially with respect to the acidogenic reactions, in order to emphasize the major metabolic routes.

The flow of carbon from polymeric biomass to methane proceeds in several successive stages. However, the degradation process is not a sequence of independent reactions, but it is characterized by a complex of mutual interactions between different microbial species[24,153]. Although the micobial populations responsible for the anaerobic digestion in different methane producing habitats are taxonomically distinct, the basic degradation pattern appears to be similar[112]. Microbial populations involved in the fermentation of cellulose in mesophilic and thermophilic environments are probably taxonomically different, but catalyze the same overall reactions. This microbial versatility allows for an extensive diversity in environmental parameters of methane producing ecosystems as to pH, osmotic pressure, temperature and substrate spectrum.

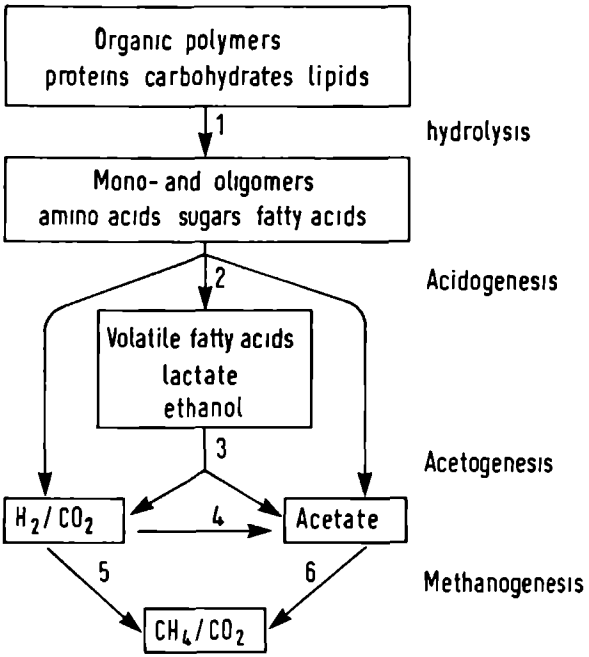



Figure 2. Anaerobic degradation of polymeric biomass to methane and the microbial groups involved. 1,2: hydrolytic and fermentative bacteria. 3: hydrogen-producing acetogenic bacteria. 4: hydrogen-consuming acetogenic bacteria. 5: hydrogenotrophic methanogens. 6: acetoclastic methanogens

## Hydrolysis

Since bacteria are unable to take up particulate organic material, the first step in anaerobic degradation consists of the hydrolysis of polymers through the action of exoenzymes to produce smaller molecules which can cross the cell barrier. Some eucaryotic microorganisms (e.g. rumen ciliates) are known to perform an intracellular hydrolysis of polymers[37]. Hydrolytic microorganisms are generally associated with the insoluble substrate[59]. Estimates of $10^8$ to $10^9$ hydrolytic bacteria per ml have been reported in mesophilic digestion of sewage sludge[91,134].

Most of the polymers possessing α-glycosidic bonds, such as starch or glycogen, are readily hydrolyzed by amylases. Pectins are easily degradable by pectinases or amylases[156], whereas proteases and peptidases are responsible for the hydrolysis of protein[22]. Cellulose, the predominant constituent of biomass has been shown to be rather resistant to hydrolysis[78,102]. The enzymes responsible for the hydrolysis of the β-(1-4)-linked units of D-glucose are referred to as cellulases. Cellulase in fact is a complex of cellulolytic enzymes, composed of exo-glucanases (exo-cellobiohydrolase), endo-glucanases and cellobiases (β-glucosidase)[47,79,89] and their synthesis is regulated by induction and catabolite repression[119]. The solube products cellobiose and glucose have been reported to be inhibitors of the cellulase complex[62,83,89]. The complete hydrolysis of insoluble cellulose requires a synergistic action of the three groups of cellulolytic enzymes[45,83,154]. Many bacteria however do not possess cellobiases, but process cellobiose and cellodextrins intracellularly by the use of specific phosphorylases[89]. The cellulase systems of *Acetovibrio cellulolyticus*[79,98,118], *Clostridium thermocellum*[30,41,101] and several cellulolytic rumen microorganisms[51,58,113] have been intensively investigated. Many differences have been reported between cellulase complexes of various species with respect to subunit composition, molecular weight, and substrate specificity and activity of isolated enzymes[89]. According to the present knowledge, cellulolytic enzyme systems appear to be almost as diverse as there are types of celluloytic microorganisms.

**Acidogenesis**

The soluble products of hydrolysis are metabolized intracellularly by a complex consortium of hydrolytic and non-hydrolytic microorganisms. Pyruvate, which is formed from hexoses or from other precursors, is a general intermediate in anaerobic fermentations and is subsequently fermented into various metabolic products. Electrons generated during pyruvate formation are utilized in the fermentation routes.

Although pure cultures of bacteria produce a variety of end products from carbohydrates[153], the main end products of acidogenesis

by mixed cultures are acetate, propionate, butyrate and $H_2/CO_2$[94,132]. Furthermore minor amounts of formate, lactate, valerate, methanol, ethanol, butanediol or acetone may be produced by fermentative microorganisms. Since volatile fatty acids (VFA) are the main products of fermentative organisms, they are usually designated as acidifying or acidogenic microorganisms.

The partial pressure of hydrogen ($PH_2$) plays an important role in controlling the proportions of various fermentation products[24,132,150]. Based on thermodynamic considerations, the production of $H_2$ from NADH becomes favourable ($\Delta G^{\circ\prime}$ negative) only at very low $PH_2$ ($< 10^{-3}$ atm)[120]. If $PH_2$ is kept below this value by $H_2$-consuming methanogens, the fermentation pattern of acidogenic microorganisms is shifted to the production of more $H_2$, $CO_2$ and acetate which may yield 4 moles of ATP per mole of hexose fermented[24,50]. However at higher $PH_2$ there is an increased tendency of the electrons of NADH to be utilized in the catabolism of pyruvate to more reduced products such as propionate, ethanol or lactate. As a result less ATP is yielded per mole of hexose fermented[94].

**Acetogenesis**

The hydrogen-producing acetogenic bacteria are responsible for the oxidation of products generated in the acidogenic phase to substrates suitable for methanogens[24,96]. For a long time it was generally believed that the products of acidogenic bacteria were directly utilized by methanogenic bacteria[9,10]. In 1967 Bryant et al[26] demonstrated that ethanol was not directly used as a substrate for methanogenesis, but was first oxidized to acetate and $H_2$ by an acetogenic species. From that time it became clear that acetogenic bacteria form an intermediate metabolic group which may deliver substrates for methanogenic bacteria.

The products of acetogenic bacteria are $H_2$, $CO_2$ and acetate. As is shown in Table 1 most of the hydrogen-generating reactions are thermodynamically unfavourable under stadard conditions. Progress of the reactions is strongly affected by $PH_2$ and may become exergonic at a relatively low partial hydrogen pressure. The effect of $PH_2$ on the free energy changes of some acetogenic reactions is shown in Fig 3.

Acetogenesis from pyruvate is thermodynamically feasible at a broad range of $PH_2$, but the oxidation of propionate, butyrate or ethanol requires a low $PH_2$. Therefore, acetogenic proton reducing bacteria performing these reactions are obligatorily coupled to hydrogen oxidizers such as methanogens[21,26,95,97] or sulfate reducing bacteria[54,112]. The physiological partnership between hydrogen producing and hydrogen consuming species in synthrophic association is termed

Table 1. Free energy change ($\Delta G°'$) of some acetogenic reactions under standard conditions

| Substrates | Products | $\Delta G°'$ (kJ.mole$^{-1}$) |
|---|---|---|
| Butyrate + $2H_2O$ | 2 Acetate$^-$+$2H^+$+$2H_2$ | +48.1 |
| Propionate + $3H_2O$ | Acetate$^-$+$HCO_3^-$+$2H^+$+$3H_2$ | +76.1 |
| Lactate + $2H_2O$ | Acetate$^-$+$HCO_3^-$+$2H^+$+$2H_2$ | -15.1 |
| Ethanol + $H_2O$ | Acetate$^-$+$H^+$+$2H_2$ | +9.6 |
| Benzoate + $7H_2O$ | 3 Acetate$^-$+$HCO_3^-$+$4H^+$+$3H_2$ | +89.7 |

'interspecies hydrogen transfer'. Methanogenesis from $H_2/CO_2$ is feasible at $PH_2$ above $2.5.10^{-6}$ atm (Fig 3). The implication of methanogens on the stochiometry and free energy change ($\Delta G°'$) of reactions involved in the catabolism of propionate is summarized in Table 2.

Recently, interspecies hydrogen transfer has been suggested to occur between sapropelic[136] and rumen ciliates[127,128,143] as hydrogen producers and methanogens as hydrogen consumers in endosymbiotic and episymbiotic association, respectively.

A special group of bacteria which are usually present in very small numbers are hydrogen consuming acetogenic bacteria[8,90,157]. These organisms require a relatively high $PH_2$ for acetate production (Fig 3). Since $PH_2$ is normally kept low due to the presence of methanogens,

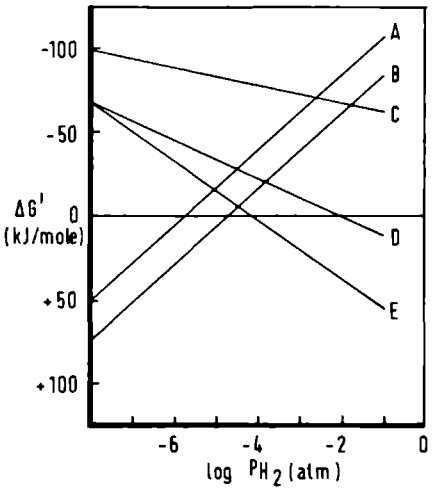



Figure 3. Effect of hydrogen partial pressure on free energy change of some reactions in anaerobic digestion. Methanogenesis from $H_2/CO_2$ (A) and acetogenesis from $H_2/CO_2$ (B), pyruvate (C) butyrate (D) and propionate (E). Calculations were based on standard values for G' at 25°C, pH7, $PCH_4$ 0.7 atm, 1 mM VFA and 34 mM $HCO_3^-$.

these organisms are probably of minor importance in the overall anaerobic digestion process. $CO_2$ reduction to acetate has been shown to account for less than 5% of the total acetate production in a cattle manure digester[90].

**Methanogenesis**

The final step in the overall anaerobic conversion of organic matter into methane and $CO_2$ is catalysed by methanogenic bacteria. Methanogens utilize only a limited number of simple substrates, comprising acetate or the $C_1$-compounds $CO_2/H_2$, formate, methanol, methylamines and CO. Although only a few species of the methanogens isolated up to now are capable of acetoclastic methane formation, about 70% of the global methane production is derived from the methyl group of acetate[93,123]. Nearly all known methanogenic species are able to produce methane from $H_2/CO_2$. Although the standard free energy change

Table 2. Effect of syntrophic association between $H_2$-producing acetogenic bacteria and methnogens on change in free energy ($\Delta G°'$) during the catabolism of propionate

| Substrates | Products | $\Delta G°'$ (kJ.mole$^{-1}$) |
|---|---|---|
| Propionate + $3H_2O$ | acetate+$HCO_3^-$+$H^+$+$3H_2$ | +76.1 (A) |
| $4H_2$+$HCO_3^-$+$H^+$ | $CH_4$+$3H_2O$ | -135.6 (B) |
| Acetate + $H_2O$ | $CH_4$+$HCO_3^-$+$H^+$ | -31.0 (C) |
| | Sum A+B | |
| Propionate +0.75 $H_2O$ | acetate + 0.75 $CH_4$+0.25 $HCO_3^-$ | -25.6 |
| | Sum A+B+C | |
| Propionate + 1.75 $H_2O$ | 1.75$CH_4$+1.25$HCO_3^-$+1.25$H^+$ | -56.6 |

in the conversion of $H_2/CO_2$ to methane is much greater as compared to acetoclastic methane formation one has to consider that $PH_2$ in anaerobic digesters or in natural environments is usually very low. Consequently the hydrogenotrophic reaction will yield considerably less energy ($\Delta G'$ about 35 kJ per mole at $PH_2$ $10^{-4}$ atm). Taking this into account, the energetics of methanogenesis from $H_2/CO_2$ or acetate probably do not differ much under non-standard conditions.

Both hydrogenotrophic and acetoclastic methanogenesis are very important in maintaining the progress of anaerobic digestion. From the previous thermodynamic considerations it is evident that methanogenic bacteria fulfill a crucial role as hydrogen scavengers in overall anaerobic digestion[6]. Hungate et al[73] reported a Km value of $10^{-3}$ mM for $H_2$ in hydrogenotrophic methane production in the rumen. The ability of methanogens to keep $PH_2$ very low, thereby pulling $H_2$-producing reactions, forms the basis for interspecies hydrogen transfer. By

keeping $PH_2$ low an accumulation of VFA, which would result in fementer performance failure due to acidification, is prevented. Acetoclastic methane formation on the other hand prevents acidification of the anaerobic environment by removal of acetate. Fermentation of organic matter in the absence of methanogens would consequently result in a rapid acidification and incomplete degradation.

If the chemical composition of the organic substrate is known, the maximum theoretical methane yield can be calculated according to the equation developed by Buswell[27]:

$$C_nH_aO_bN_c + \left(n - \frac{a}{4} - \frac{b}{2} + \frac{3c}{4}\right) H_2O \longrightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8}\right) CH_4 +$$

$$\left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + \frac{3c}{8}\right) CO_2 + cNH_3$$

During the anaerobic decomposition of complex organic matter, about 90% of the substrate energy is retained in the methane that is produced[24]. The complete conversion of organic material to $CH_4$ and $CO_2$ has been subject of recent reviews[24,89,92,112,159].

## RATE LIMITING STEPS

Since anaerobic digestion of organic matter is a multistep process which involves the successive action of several metabolicly diverse populations of microorganisms, the overall rate of substrate conversion is determined by the kinetic characteristics of the slowest step. Which of the individual steps is rate-limiting is mainly dependent on the composition of the substrate.

During the digestion of refractory insoluble substrates, such as cellulose, the hydrolysis step has been reported to govern the overall degradation rate[42,102,114]. The rate of hydrolysis is determined by both microbial constraints (e.g. generation time[102], cellulase production) and physical and chemical characteristics of the substrate (e.g. crystallinity of cellulose, degree of association with lignin,

surface area/particle size ratio)[73,87].

If soluble organic components are the main substrates for anaerobic, digestion, acetogenesis and methanogenesis have been identified as rate-limiting steps[48,77,85]. This is a direct consequence of the long generation times of most Hydrogen-producing acetogenic bacteria and acetoclastic methanogens[64,81]. The low growth yield and long doubling times reported for these organisms are due to the low free energy changes for acetogenesis and methanogenesis at physiological conditions[24]. This has major implications on the anaerobic teratment of organic waste in a digester. Methanogenic bacteria in anaerobic digesters may exhibit mass doubling times of more than 30 days[57,137]. As a consequence average residence times reported for completely stirred tank reactors (CSTR) are in the range of 20-40 days, resulting in low volumetric loading rates.

Recent advances in the technology of anaerobic digestion of soluble waste and understanding of the complex process have resulted in drastic improvements of digester performance. The general strategy to counteract the rate limitation of conventional anaerobic digesters (CSTR) treating waste water is based on the increase of microbial biomass retention at relatively short hydraulic residence time. Methods to improve microbial biomass retention are all based on the general phenomenon that the bacteria attach readily to surfaces and to one another.

The first retained biomass reactor that was developed was the anaerobic contact reactor[121]. More advanced desings were developed during the last 20 years and include the anaerobic filter[155], the upflow anaerobic sludge blanket[86] which in fact is an improvement of the earlier developed clarigester[125], the fluidized and expanded bed reactors[56,75,130] and downflow stationary fixed film reactors[100,139].

The different designs of retained biomass reactors, all based on the concept of immobilization of slowly growing organisms have been reviewed recently[29,120,138]. The different treatment methods all have their own specific benefits and drawbacks and they are limited to the treatment of certain types of waste waters. Due to the accumulation and immobilization of microbial biomass, relatively high volumetic loading rates of low strength waste waters can be realised. For optimal application and start-up of these systems more information is

required on the conditions that stimulate attachment of anaerobic bacteria to various supports[7] or the formation of granules with excellent settling properties in sludge blanket reactors[40,63].

While major advances have been made in the anaerobic treatment of soluble wastes, digestion of solid organic wastes is still performed in low-rate CSTR. Immobilization of microbial cells on supports may not be applicable in solid waste treatment, since an optimal contact between the solid substrate and the microorganisms should be maintained. During the digestion of particulate waste materials the microorganisms in fact are immobilized on the substrate particles and therefore increased biomass retention may be realized by selective retainment of the solid substrate whereas the retention time of rapidly digested soluble waste components may be reduced. This mode of operation has been applied to the digestion of animal manures by means of upflow or horizontal plug flow digester designs[28,29].

An alternative means of accelerating the decomposition of cellulosic residues is to make the substrate more accessible to enzymic attack by means of pretreatment. The goal of pretreatments is to break-up the lignin-cartohydrate complex in order to increase the degree and the rate of cellulose digestion. A variety of pretreatment methods have been developed including physical, chemical and biological methods such as gamma irradiation, ball milling, steam explosion, acid and alkali treatments or micobial treatment with white-rot and brown-rot fungi[53,80,99,103,104]. Most of the pretreatments have been developed with the primary goal to valorize low quality forages as an animal feed especially for ruminants[99], but recently they were also applied to lignocellulosic wastes before anaerobic digestion[103,104]. It is not yet clear whether the additional costs of pretreatment will be recovered in reduced costs of digestion.

A more direct means of improving cellulose degradation would be by the application of microorganisms or microbial communities exhibiting an enhanced cellulolytic activity[49,60,131]. In contrast to the inefficient degradation of solid waste materials in current digesters, the anaerobic decomposition of lignocellulosic materials in certain natural microbial ecosystems is known to proceed at high rates. The termite hindgut or the forestomach of ruminants may be considered as high-rate natural digesters which operate at solid residence times of only one

Table 3. Performance data of some natural and artificial anaerobic microbial ecosystem

| Type of reactor | Substrate | loading rate g VS[a] $l^{-1}$ $d^{-1}$ | retention time (d) | conversion (%) | reference |
|---|---|---|---|---|---|
| CSTR[b] | domestic refuse | 1-2 | 20-40 | 50 | 140 |
| CSTR | pig manure | 3-6 | 10-20 | 30-40 | 141 |
| Two-phase | tomato plants | - | 14-21 | 40 | 61 |
| UASB[c] | waste water | 15-18 | 0.13-0.33 | 95 | 86 |
| Fluidized bed | waste water | 20-60 | 0.04-0.08 | 90 | 56 |
| Termite hindgut | lignocellulose | 35-70 | 0.5-1 | 60 | |
| rumen | grass | 50-100 | 1-2 | 40-70 | |

[a] Volatile solids

[b] Completely stirred tank reactor

[c] Upflow anaerobic sludge blanket

or two days (Table 3). These animals and all other herbivores have evolved a symbiotic interrelationship with microorganisms which enable them to convert huge amounts of structural plant polysaccharides that cannot be degraded by animal digestive processes. Herbivorous animals rely on the gut microorganisms to convert these polysaccharides into products which can be utilized for energy and growth. The rate of hydrolysis and subsequent acid formation by intestinal microorganisms may be up to 20-50 times as high as compared to artificial anaerobic systems treating cellulosic waste materials (Table 3). Because of their high cellulolytic activity and relatively short generation times, rumen microorganisms possess an enormous biotechnological potential in the application to anaerobic decomposition of cellulosic residues[49].

The rumen is an open continuous cultivation system of microorganisms in which conditions suitable for microbial growth are provided by the ruminant. The rumen ecosystem is characterized by an almost constant supply of plant material, saliva and water, a constant temperature of 39°C, an almost neutral pH (6-7), a low oxidation-reduction potential and a differential removal rate of solids and liquids[33,68]. These conditions favor the growth of a large and complex microbial population which is responsible for the conversion of structural plant fibres. Microorganisms involved in ruminant fermentation processes include a great variety of bacteria, many ciliate protozoa not found elsewhere in nature, flagellates and phycomycete fungi[12,16,34,68,109]. From the discovery of ciliate protozoa in rumen content in 1843[52], it took more than a century before the function of microorganisms in ruminant metabolism was elucidated[44,65,115].

In contrast to the anaerobic degradation pattern depicted in Fig 2, which represents an overall digestion of organic matter to $CH_4$ and $CO_2$, the degradation of plant material in the rumen involves only the stages of hydrolysis and acidogenesis. Hydrogen producing acetogenic bacteria which catabolize VFA and acetoclastic methanogenic bacteria do not grow rapidly enough to be maintained at the relatively short residence time of the feed material in this ecosystem[70]. Consequently, acetate (56-70%), propionate (17-29%), butyrate (9-19%) and minor amounts of longer-chained saturated fatty acids are the predominant fermentation products in the rumen[23,68,152]. These products are absorbed from the digestive tract and serve as a major energy source for the ruminant. Hydrogen is an important intermediate in rumen fermentation and is utilized by methanogens for the reduction of $CO_2$ to $CH_4$. The hydrogenotrophic methanogens in the rumen have a high affinity for $H_2$[72], and by its consumption they enable the production of more oxidized fermentation products (acetate) by other microorganisms. Although methane production represents a loss of potential substrate energy by the animal, it results in higher ATP yields and consequently increased growth of the microorganisms involved in rumen fermentation.

Fermentation of cellulose and other complex substrates in the rumen

proceeds by the interaction of various microbial species. Pure cultures of rumen microorganisms may produce substantial amounts of lactate, ehtanol, formate, hydrogen or succinate, products usually not found in high amounts in the rumen[18,116,151,153]. Because of the low $PH_2$ created by methanogens ethanol, lacate and succinate are metabolized in the rumen.

## RUMEN MICROORGANISMS AND THEIR RELATIVE IMPORTANCE

The rumen contains a couple of hundred different species of microorganisms including bacteria ($10^{11}$-$10^{12}$cells.ml$^{-1}$), protozoa ($10^{5}$-$10^{6}$cells.ml$^{-1}$) and fungi which are firmly attached to the solid substrate during its degradation[3]. Within only a few minutes plant particles ingested by the ruminant were colonized by bacteria[32] and protozoa[4,13] and within 2 h by fungi[14,107,108]. Invasion by microorganisms was shown to start preferentially at damaged tissues[17,84,144]. The physical coupling of the microorganisms to their substrate enables them to exploit their hydrolytic enzyme activities in an optimal way. Moreover, attachment of microbial cells to the solid digesta provides a means of increasing microbial biomass retention in the rumen, since solid residence time has been shown to be much longer than retention of liquids[144].

### Rumen bacteria

Only a few of the predominant species of microorganisms that inhabit the rumen produce cellulase. The predominant cellulolytic bacteria in the rumen appear to be *Ruminococcus albus*[67], *Bacteroides succinogenes*[66], *Ruminococcus flavefaciens*[122] and *Butyrovibrio fibrisolvens*[25], all studied in pure culture. Of these species *Bacteroides succinogenes* is probably the most active in the degradation of recalcitrant forms of cellulose[87]. The cellulase complexes of various cellulolytic rumen bacteria have been studied and seem to differ markedly between different species[89].

The products of cellulose hydrolysis are subsequently metabolized by

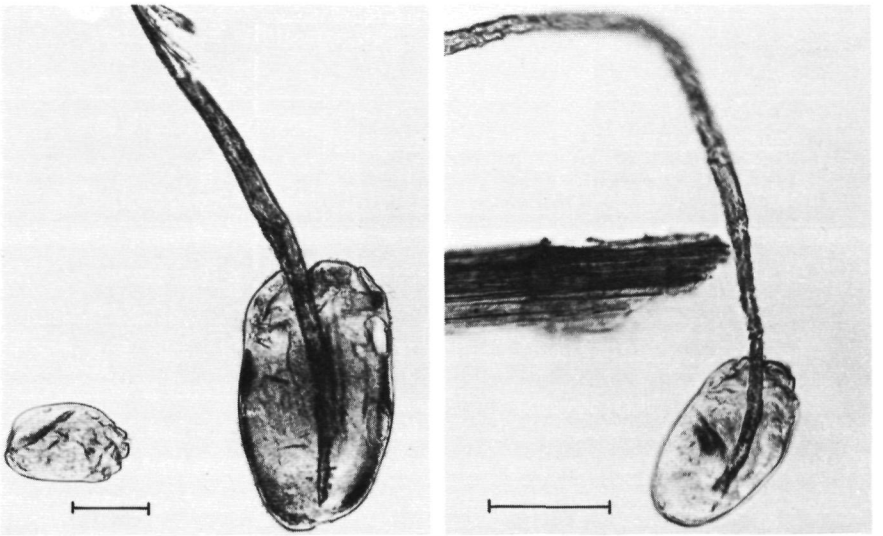


Figure 4. Bright field micrograph of rumen ciliates engulfing plant fibres. The bar represents 50 µm.

both cellulolytic and non-cellulytic fermentative rumen bacteria.

**Rumen ciliate protozoa**

The ciliates in the rumen belong to the families of *Ophryoscolecidae* and *Isotrichidae*. These ciliates are highly specialized for growth in the rumen ecosystem, since they are not found elsewhere. Because of the large dimensions of rumen ciliates (25-200 µm), their morphology has been well documented[35,68]. Microscopic observations have demonstrated that many rumen ciliates are able to engulf starch granules or plant fibres (Fig 4)[2,5,15,36]. Moreover, a wide range of hydrolytic enzymes necessary for the degradation of plant cell wall polysaccharides have been demonstrated in extracts of rumen ciliate protozoa[19,38,146,147,148]. Cellulolytic activity recently was also demonstrated in a culture of the rumen ciliate *Polyplastron multivesiculatum* free of cellulolytic bacteria[20]. Although the active involvement of rumen ciliates in plant cell wall degradation has been established, the exact role and metabolic activity of these organisms is not fully understood. It has been demonstrated in many studies that

ruminants can survive without the presence of ciliates but fibre degradation and weight gain were usually lower in defaunated animals[1,76,82,149]. Based on literature results, Demeyer reported that rumen protozoa account for 34% of the total rumen microbial fibre digestion[151].

### Phycomycete fungi

Besides ciliates also flagellate protozoa have been observed in the rumen, but their number is probably much lower[68]. Recently, however some of the rumen flagellates were identified as zoospores of the phycomycete fungi *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis*[105,106,108]. The life cycle of these phycomycete fungi consists of a motile zoospore stage free in rumen fluid and a non-motile vegetative reproductive stage which is firmly attached to feed particles[12,108,110]. Under favourable conditions up to 8% of the microbial biomass may consist of Phycomycete fungi[109]. Although these organisms are known to ferment plant cell wall polysaccharides in pure cultures[110], their share in the overall digestion of plant fibre in the rumen is not yet established.

## OUTLINE OF THE THESIS

Considering the fact that rumen microorganisms exhibit extremely high cellulase activities and relatively short mass doubling times, it is obvious that an application of these organisms to the anaerobic treatment of cellulosic residues might significantly enhance the rate and the economical feasibility of the process. This thesis describes the development of a high-rate artificial rumen digester and its application to the degradation of mainly (ligno-)cellulosic wastes.

Chapter 2 describes the long-term cultivation of rumen microorganisms, including ciliates, in an artificial rumen digester. The role of ciliates in the overall rumen fermentation process is discussed in Chapter 3.

In order to realize a complete conversion of cellulose to $CH_4$ and

$CO_2$, the artificial rumen digester was coupled to an UASB-type methane reactor thereby creating a high-rate two-phase process which is referred to as the 'rumen derived anaerobic digestion' (RUDAD-) process (Chapter 4). Chapter 5 and 6 deal with the application of the artificial rumen digester to the anaerobic digestion of various organic waste materials. The stability of the RUDAD-process is demonstrated during a long-term digestion period with a cellulosic fraction of domestic refuse as a digester feed (Chapter 7).

A technological simplification of the RUDAD-process based on the microbial immobilization is presented in Chapter 8.

## REFERENCES


1 Abou-Akkada AR, El-Shazly K (1964) Effect of absence of ciliate protozoa from the rumen on microbial activity and growth of lambs. Appl Microbiol 12: 384-390

2 Akin DE, Amos HE (1979) Mode of attack on orchard grass leaf blades by rumen protozoa. Appl Environ Microbiol 37: 332-338

3 Akin DE, Barton FE (1983) Rumen microbial attachment and degradation of plant cell walls. Fed Proc 42. 114-121

4 Amos HE, Akin DE (1978) Rumen protozoal degradation of structurally intact forage tissues. Appl Environ Microbiol 36: 513-522

5 Amos HE, Akin DE (1978) Rumen protozoal degradation of structurally intact forage tissues. Appl Environ Microbiol 36: 513-522

6 Archer DB (1983) The microbiological basis of process control in methanogenic fermentation of soluble wastes. Enzyme Microb Technol 5: 162-169

7 Ash SG (1979) Adhesion of microorganisms in fermentation processes. In: Ellwood DC, Melling J, Rutter P (eds) Adhesion of microorganisms to surfaces. Soc Gen Microbiol, Academic Press, London, p 57-86

8 Balch WJ, Schoberth S, Tanner S, Wolfe R (1977) Acetobacterium a new genus of hydrogen-oxidizing, carbondioxide-reducing, anaerobic bacteria. Int J Syst Bacteriol 27: 355-361

9 Barker HA (1940) Studies upon the methane fermentation, IV The isolation and culture of Methanobacterium omelianskii. Antonie van Leeuwenhoek J Microbiol 6: 201-220

10 Barker HA (1956) Bacterial fermentations. John Wiley and Sons Inc, New York

11 Bassham JA (1975) General considerations. Biotechnol Bioeng Symp 5: 9-19

12 Bauchop T (1979) Rumen anaerobic fungi of cattle and sheep. Appl Environ Microbiol 38: 148-158

13 Bauchop T (1979) The rumen ciliate Epidinium in primary degradation of plant tissues. Appl Environ Microbiol 37: 1217-1223
14 Bauchop T (1979) The rumen anaerobic fungi: colonizers of plant fibre. Ann Rech Vet 10: 246-248
15 Bauchop T (1980) Scanning electron microscopy in the study of microbial digestion of plant fragments in the gut. In: Elwood DC, Hedges JN, Latham MJ, Lynch JM, Slater JH (eds) Contemporary microbial ecology. Acad Press, London, New York, p305-326
16 Bauchop T (1981) The anaerobic fungi in rumen fibre digestion. Agric Environ 6: 339-348
17 Bauchop T, Clarke RT (1976) Attachment of the ciliate Epidinium crawley to plant fragments in the sheep rumen. Appl Environ Microbiol 32: 417-422
18 Bauchop T, Mountfort DO (1981) Cellulose fermentation by a rumen anaerobic fungus in both the absence and presence of rumen methanogens. Appl Environ Microbiol 42: 1103-1110
19 Bonhomme-florentin A (1975) Activité cellulotytique de ciliés entodiniomorphes. J Protozoal 22: 447-451
20 Bonhomme A, Fonty G, Foglietti MJ, Robic D, Weber M (1986) Endo-1,4-B-glucanase and B-glucosidase of the ciliate Polyplastron multivesiculatum free of cellulolytic bacteria. Can J Microbiol 32: 219-225
21 Boone DR, Bryant MP (1980) Propionate degrading bacterium, Syntrophobacter wolinii sp nov gen nov, from methanogenic ecosystems. Appl Environ microbiol 40: 626-632
22 Brock FM, Forsberg CW, Buchanan-Smith JG (1982) Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl Environ Microbiol 44: 561-569
23 Bryant MP (1970) Normal flora - rumen bacteria. Am J Clin Nutr 23: 1440-1450
24 Bryant MP (1979) Microbial methane production - Theoretical aspects. J Anim Sci 48: 193-201
25 Bryant MP, Small N (1956) The anaerobic monotrichous butyric acid-producing curved rod shaped bacteria of the rumen. J Bacteriol 76: 16-21
26 Bryant MP, Wolin EA, Wolin MJ, Wolfe RS (1967) Methanobaccillus omelianskii, a symbiotic association between two species of bacteria. Arch Microbiol 59: 20-31
27 Buswell AM (1930) Production of fuel gas by anaerobic fermentations. Ind Eng Chem 22: 1168-1172
28 Callander IJ (1982) The development of the tower fermenter for anaerobic digestion. PhD thesis, University of Sydney, Australia
29 Callender IJ, Barford JP (1983) Recent advances in anaerobic digestion technology. Process Biochem 18: 24-30
30 Carreira L, Ljungdahl LG (1983) Production of ethanol from biomass using anaerobic thermophilic bacteria. In: Wise D (ed) liquid fuel developments. CRC press, Boca Raton, Florida, p 1-29
31 Caskey WH, Tiedje JM (1980) The reduction of nitrate to ammonium by a Clostridium sp isolated from soil. J Gen Microbiol 119 : 217-223
32 Cheng KJ, Akin DE, Costerton JW (1977) rumen bacterial interac-

tion with particulate dietary components and response to dietary variation. Fed Proc 36: 193-197
33 Clarke RTJ (1977) Methods for studying gut microbes. In: Clarke RTJ, Bauchop T (eds) Microbial ecology of the gut. Acad Press, New York, p 1-33
34 Clarke RTJ (1977) Protozoa in the rumen ecosystem. In: Clarke RTJ, Bauchop T (eds) Micorbial ecology of the gut. Acad Press, New York, p251-275
35 Coleman GS (1978) rumen entodinomorphid protozoa. In: Tayler AER, Baker JR (eds) Methods of cultivating parasites in vitro. Acad Press, London p39-54
36 Coleman GS (1980) rumen ciliate protozoa. Adv Parasitol 18: 121-174
37 Coleman GS (1983) The cellulolytic activity of thirteen species of rumen entodiniomorphid protozoa. J Protozool 30: 36A
38 Coleman GS (1986) The metabolism of rumen ciliate protozoa. FEMS Microbiology Reviews 39: 321-344
39 Demeyer DI (1981) Rumen microbes and digestion of plant cell walls. Agric Environ 6: 295-337
40 de Zeeuw W (1984) Acclimatization of anaerobic sludge for UASB-reactor start-up. PhD thesis, Agricultural University Wageningen, the Netherlands
41 Duong TVC, Johnson EA, Demain AL (1983) Thermophilic, anaerobic and cellulolytic bacteria. In: Wiseman A (ed) Topics in Enzyme and fermentation technology 7. John Wiley, New York, p 156-195
42 Eastman JA, Ferguson JF (1981) Solubilization of particulate organic cabon during the acid phase of anaerobic digestion. J Water Pollut Control Fed 53: 352-366
43 Ehalt DH (1976) The atmospheric cycle of methane. In: Schlegel HG, Gottschalk G, Pfennig N (eds) Microbial production and utilization of gases. Goltze KG, Göttingen, p 13-22
44 Elsden SR, Hitchkock MWS, Marshall RA, Phillipson AT (1946) Volatile acids in the digesta of ruminants and other animals. J Exp Biol 22: 191-202
45 Eriksson KE, Wood TM (1984) Biodegradation of cellulose In: *Higuchi T (ed) Biosynthesis and biodegradation of wood components.* Academic press, New York, p 469-503
46 Fenchel TM, Jorgensen BB (1977) Detritus food chains of aquatic ecosystems: the role of bacteria. Adv Microb Ecol 1:1-58
47 Garcia-Martinez DV, Shinmyo A, Midia A, Demain AL (1980) Studies on cellulase production by Clostridium thermocellum. Eur J Appl Microbiol Biotechnol 9: 189-197
48 Ghosh S, Pohland FG (1974) kinetics of substrate assimilation and Product formation in anaerobic digestion. J Water Pollut Control Fed 46: 748-759
49 Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986) Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of ligno-cellulosic waste materials. Appl Microbiol Biotechnol 25: 155-162
50 Gottschalk G (1979) Bacterial metabolism. Springer Verlag, New York
51 Groleau D, Forsberg CW (1981) Cellulolytic activity of the rumen bacterium Bacteroides succinogenes. Can J Microbiol 27: 517-530

52 Gruby D, Delafond D (1843) Recherches sur des animalcules qui se développent en grand nombre dans l'estomac et les intestins pendant la digestion des herbivores et des carnivores. Cr Acad Sci 17: 1304-1308

53 Han YW, Pence JW, Anderson PW (1975) Dioxane pretreatment to improve microbial digestibility of cellulosic fibres. Appl Microbiol 29: 708-709

54 Hatchikian EC, Chaigneau M, LeGall J (1976) Analysis of gas production by growing cultures of three species of sulfate reducing bacteria. In: Schlegel HG, Gottschalk G, Pfennig N (eds) Microbial production and utilization of gases. Goltze KG, Göttingen, p109-118

56 Heijnen JJ (1983) Development of a high-rate fluidized bed reactor. In: Proc Eur Symp Anaerobic waste water treatment, Nov 1983, Noordwijkerhout, the Netherlands, p 283-291

57 Henze M, Harremoes P (1982) Literature review: anaerobic treatment of waste water in fixed film reactors. In: Proceedings of the international association on water pollution, research seminar on anaerobic treatment of waste water in fixed film reactors, Copenhagen. Univ of Denmark, Copenhagen

58 Hiltner P, Dehority BA (1983) Effect of soluble carbohydrates on digestion of cellulose by pure cultures of rumen bacteria. Appl Environ Microbiol 46: 642-648

59 Hobson PN, Wallace RJ (1982) Microbial ecology and activities in the rumen: part I. CRC crit rev Microbiol 9: 165-225

60 Hobson PN, Reid GW, Sharma VK (1984) Anaerobic conversion of agricultural wastes to chemicals or gases. In: Ferrero GL, Ferranti MD, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste. Elsevier Applied Science Publishers, London, New York, p369-381

61 Hoffenk G (1983) Anaerobic digestion of solid organic wastes, process selection and pilot plant research. Proc Eur Symp Anaerobic waste water treatment, Noordwijkerhout, the Netherlands, p475-491

62 Howell JA, Stuck JD (1975) kinetics of solca floc cellulose hydrolysis by Trichoderma viride cellulase. Biotechnol Bioeng 17: 873-893

63 Hulshoff Pol L, Dolfing J, de Zeeuw W, Lettinga G (1982) Cultivation of well adapted pelletized methanogenic sludge. Biotechnol Lett 4: 329-332

64 Huser BA (1981) Methanbildung aus Acetat. PhD thesis. ETH Zürich, no. 6750

65 Hungate RE (1942) The culture of Eudiplodinium neglectum with experiments on the digestion of cellulose. Biol Bull Woods Hole 83: 303-319

66 Hungate RE (1950). The anaerobic mesophilic cellulolytic bacteria. Bacteriol Rev 14: 1-50

67 Hungate RE (1957) Microorganisms in the rumen of cattle fed a constant ration. Can J Microbiol 3: 289-311

68 Hungate RE (1966) The rumen and its microbes. Academic Press, New York

69 Hungate RE (1975) The rumen microbial ecosystem. Annu Rev Ecol Syst 6: 39-66

70 Hungate RE (1976) The rumen fermentation. In: Schlegel HG, Gottschalk G, Pfennig N (eds) Microbial production and utiliza-

tion of gases, Goltze KG, Gōtingen, p 119-124
71 Hungate RE (1982) Methane formation and cellulose digestion - biochemical ecology and microbiology of the rumen ecosystem. Experientia 38: 189-192
72 Hungate RE, Doughterty RW, Bryant MP, Cello RM (1952). Microbiological and physiological changes associated with acute indigestion in sheep. Cornell Vet 42: 423-449
73 Hungate RE, Smith W, Bauchop T, Yu I, Rabinowitz JC (1970) Formate as an intermediate in the bovine rumen fermentation. J Bacteriol 102: 389-397
74 Iannotti EL, Kafkewitz P, Wolin MJ, Bryant MP (1973) Glucose fermentation products of Ruminococcus albus grown in continuous culture with Vibrio succinogenes: changes caused by interspecies transfer of $H_2$. J Bacteriol 114: 1231-1240
75 Jewell WJ, Switzenbaum MS, Morris JW (1981) Municipal waste water treatment with the anaerobic attached microbial film bed process. J Water Pollut Control Fed 53: 482-490
76 Jouany JP, Senaud J (1979) Role of rumen protozoa in the digestion of food cellulosic materials. Ann Rech Vet 10: 261-263
77 Kaspar HF, Wuhrmann K (1978) kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. Appl Environ Microbiol 36: 1-7
78 Khan AW (1977) Anaerobic degradation of cellulose by mixed culture. Can J Microbiol 23: 1700-1705
79 Khan AW (1980) Cellulolytic enzyme system of Acetivobrio cellulolyticus, a newly isolated anaerobe. J Gen Microbiol 121: 499-502
80 Kirk TK, Moore WE (1972) Removing lignin from wood with white-rot fungi and digestibility of resulting wood. Wood fiber 4: 72-79
81 Koch M, Dolfing J, Wuhrmann K, Zehnder AJB (1983) Pathways of propionate degradation by enriched methanogenic cultures. Appl Environ Microbiol 45: 1411-1414
82 Kurihara Y, Takechi T, Shibata F (1978) Relationship between bacteria and ciliate protozoa in the rumen of sheep fed on a purified diet. J Agric Sci Camb 90: 373-381
83 Ladisch MR, Lin KW, Voloch M, Tsao GT (1983) Process considerations in the enzymatic hydrolysis of biomass. Enzyme Microb Technol 5: 81-102
84 Latham MJ, Brooker BE, Pettipher GL, Harris PJ (1978) Ruminococcus flavefaciens cell coat and adhesion to cotton cellulose and to cell walls in leaves of perennial ryegrass (Lolium perenne). Appl Environ Microbiol 35: 156-165
85 Lawrence AW, McCarty PJ (1969) kinetics of methane fermentation in anaerobic treatment. J Water Pollut Control Fed 41: 1-17
86 Lettinga G, van Velsen AFM, Hobma SW, de Zeeuw W, Klapwijk A (1980) Use of the Upflow sludge blanket (USB) reactor concept for biological waste water treatment, especially for anaerobic treatment. Biotechnol Bioeng 22: 699-734
87 Lin KW, Patterson JA, Ladisch MR (1985) Anaerobic fermentation: microbes from ruminants. Enzyme Microb Technol 7: 98-107
88 Lipinsky ES (1981) Chemicals from biomass: Petrochemical substitution options. Science 212: 1465-1471
89 Ljungdahl LG, Eriksson KE (1985) Ecology of microbial cellulose degradation. Adv Microb Ecol 8: 237-299

90 Mackie RI, Bryant MP (1981) Metabolic activity of fatty acid oxidizing bacteria and the contribution of acetate, propionate, butyrate and $CO_2$ to methanogenesis in cattle waste at 40° and 60°C. Appl Environ Microbiol 41: 1363-1373

91 Mah RA, Sussman L (1967) Microbiology of anaerobic sludge fermentation; I Enumeration of the non-methanogenic anaerobic bacteria. Appl Microbiol 16: 358

92 Mah RA, Ward DM, Baresi L, Glass TL (1977) Biogenesis of methane. Annu Rev Microbiol 31: 309-341

93 McCarty PL (1964) The methane fermentation. In: Henkelekian H, Dondero NC (eds) Principles and applications in aquatic microbiology. John Wiley and Sons Inc, New York, p314

94 McInerney MJ, Bryant MP (1981) Review of methane fermentation fundamentals. In: Wise DL (ed) Fuel gas production from biomass. Chemical Rubber Co Press Inc, West Palm Beach, Florida p 26-40

95 McInerney MJ, Bryant MP, Hespell RB, Costerton JW (1981) Syntrophomonas wolfei gen nov sp nov, an anaerobic syntrophic fatty acid oxidizing bacterium. Appl Environ Microbiol 41: 1029-1039

96 McInerney MJ, Bryant MP, Pfennig N (1979) Anaerobic bacterium that degrades fatty acids ($C_4$-$C_8$) in syntrophic association with methanogens. Arch Microbiol 122: 129-136

97 McInerney MJ, Mackı RI, Bryant MP (1981). Syntrophic Association of a Butyrate-degrading bacterium and Mehtanosarcina enriched from bovine rumen fluid. Appl Environ Microbiol 41: 826-828

98 Mackenzie CR, Bilous D (1982) location and kinetic properties of the cellulase system of Acetovibrio cellulolyticus. Can J Microbiol 28: 1158-1164

99 Millet MA, Baker AJ, Satter LD (1975) Pretreatments to enhance chemical, enzymic and microbiological attack of cellulosic materials. Biotechnol Bioeng Symp 5: 193-219

100 Murray WD, van den Berg L (1981) Effect of support material on the development of microbial fixed films converting acetic acid to methane. J Appl Bacteriol 51: 257-265

101 Ng TK, Zeikus JG (1981) Purification and characterization of an endoglucanase (1,4-B-D-glucan glucanohydrolase) from Clostridium thermocellum. Biochem J 199: 341-350

102 Noike T, Endo G, Chang JE, Yaguchi JI, matsumoto JI (1985) Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion. Biotechnol Bioeng 27: 1482-1489

103 Oi S, Tanaka T, Yamamoto T (1981) Methane fermentation of coffee grounds and some factors to improve the fermentation. Agric Biol Chem 45: 871-878

104 Oi S, Yamanaka H, Yamamoto T (1980) Methane fermentation of bagasse and some factors to improve the fermentation. J Ferm Technol 58: 367-372

105 Orpin GC (1975) Studies on the rumen flagellate Neocallimastix frontalis. J Gen Microbiol 91: 249-262

106 Orpin GC (1976) Studies on the rumen flagellate Spaeromonas communis. J Gen Microbiol 94: 270-280

107 Orpin CG (1977) Invasion of plant tissue in the rumen by the flagellate Neocallimastix frontalis. J Gen Microbiol 98: 423-430

108 Orpin GC (1977) The rumen flagellate Piromonas communis: its life

history and invasion of plant material in the rumen. J Gen Microbiol 99: 107-117
109 Orpin GC (1984) The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim Fd Sci Technol 10: 121-143
110 Orpin GC, Letcher AJ (1979) Utilization of cellulose, starch, xylan and other hemicelluloses by the rumen phycomycete Neocallimastix frontalis. Curr Microbiol 3: 121-124
111 Peck HD Jr (1984) Physiological diversity of the sulfate bacteria. In: Strohl WR, Tuovinen OH (eds) Microbial chemoautotrophy. Ohio State University Press, Columbus, p 309-335
112 Peck HD Jr, Odom M (1981) Anaerobic fermentations of cellulose to methane. In: Hollaender A (ed) Trends in biology of fermentations for fuels and chemicals. Plenum Press, New York, p375-395
113 Pettipher GL, Latham MJ (1979) Characteristics of enzymes produced by Ruminococcus flavefasiens which degrade plant cell walls. J Gen Microbiol 110: 21-27
114 Pfeffer JT (1974) Temperature effects on anaerobic fermentation of domestic refuse. Biotechnol Bioeng 16: 771-787
115 Phillipson AT (1947) The role of the microflora of the alimentary tract of herbivores with special reference to ruminants, 3. Fermentation in the alimentary tract and metabolism of the derived fatty acids. Nutr Abstr Rev 17:12
116 Prins RA, Clarke RTJ (1980) Microbial ecology of the rumen. In: Ruckebusch Y, Thivend P (eds) Digestive physiology and metabolism in ruminants, Proc 5th Int Symp Rum Physiol. MTP Press, Lacaster, England p 179-204
117 Rasmussen RA, Khalil MAK (1983) Global production of methane by termites. Natura 301: 704-705
118 Saddler JN, Khan AW (1981) Cellulolytic enzyme system of Acetovibrio cellulolyticus. Can J Microbiol 27: 288-294
119 Saddler JN, Khan AW, Martin SM (1980) Regulations of cellulase synthesis in Acetovibrio cellulolyticus. Microbios 28: 97-106
120 Sahm H (1984) Anaerobic wastewater treatment. In: Fiechter A (ed) Advances in Biochemical Engineering Biotechnology, 29, Springer, Berlin, Heidelberg, New York, Tokyo, p83-115
121 Schroepfer GJ, Fuller WJ, Johnson AS, Ziemke NR, Anderson JJ (1955) The anaerobic contact process as applied to packinghouse wastes. Sewage Ind Wastes 27: 460-486
122 Sijpesteijn AK (1951) On Ruminococcus flavefaciens, a cellulose decomposing bacterium from the rumen of sheep and cattle. J Gen Microbiol 5: 869-879
123 Smith PH, Mah RA (1965) kinetics of acetate metabolism during sludge digestion. Appl Microbiol 14: 368-371
124 Stafford DA, Hawkes DL, Horton R (1980) Methane production from waste organic matter. CRC press, BOCA Raton, Florida
125 Stander GJ (1967) Treatment of wine distillery wastes by anaerobic digestion. In: Proc 22nd Purdue Industrial waste conference, May 1967. Ann Arbor Science Publishers, Ann Arbor MI, p 892-907
126 Stephens GR, Heichel GH (1975) Agricultural and forest products as sources of cellulose. Biotechnol Bioeng Symp 5: 27-42
127 Stumm CK, Gijzen HJ, Vogels GD (1982) Association of methanogenic bacteria with ovine rumen ciliates. Br J Nutr 47: 95-99

128 Stumm CK, Zwart KB (1986) Symbiosis of protozoa with hydrogen - utilizing methanogens. Microbiol Sci 3: 100-105
129 Swift MJ (1977) The ecology of wood decomposition. Sci Prog Oxf 64: 175-199
130 Switzenbaum MS, Jewell WJ (1980) Anaerobic attached-film expanded-bed reactor treatment. J Water pollut Control Fed 52: 1953-1965
131 Taya M, Ohmiya K, Kobayashi T, Shimizu S (1983) Enhancement of cellulose digestion by mutants from an anaerobe, Ruminococcus albus. J Ferment Technol 61: 197-199
132 Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41: 100-180
133 Thompson NS (1983) Hemicellulose as a biomass resource. In: Soltes EJ (ed) Wood and Agricutral residues. Acamdemic Press, New York, p 101-119
134 Toerien DF, Siebert MF (1967) A method for the enumeration and cultivation of anaerobic acid-forming bacteria in digesting sludge. Water Res 1: 397-404
135 Tsao GT, Ladisch MR, Ladisch C, Hsu TA, Dale B, Chou T (1978). Fermentation substrates from cellulosic materials: production of fermentable sugars from cellulosic materials. Annu Rep Ferment Proc 2: 1-21
136 van Bruggen HJA (1986) Methanogenic bacteria as endosymbionts of sapropelic protozoa. PhD Thesis, University of Nijmegen, The Netherlands
137 van den Berg L (1977) Effect of temperature on growth and activity of a methanogenic culture utilizing acetate. Can J Microbiol 23: 898-902
138 van den Berg L (1984) Developments in methanogenesis from industrial waste water. Can J Microbiol 30: 975-990
139 van den Berg L, Kennedy KJ (1980) Support materials for stationary fixed film reactors for high-rate methanogenic fermentations. Biotechnol Lett 3: 165-170
140 van der Vlugt AJ, Rulkens WH (1984) Biogas production from a domestic waste fraction. In: Ferrero GL, Ferranti MP, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste. Elsevier Applied Science Publishers, London, New York, p245-250
141 van Velsen AFM (1981) anaerobic digestion of piggery waste. PhD Thesis, Agricultural University of Wageningen, The Netherlands
142 Vogels GD (1979) The global cycle of methane. Antonie Leeuwenhoek J Microbiol Serol 45:347-352
143 Vogels GD, Hoppe WF, Stumm CK (1980) Association of methanogenic bacteria with rumen ciliates. Appl Environ Microbiol 40: 608-612
144 Weller RA, Pilgrim AF (1974) Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Br J Nutr 32: 341-352
145 Whittaker RH, Likens GE (1973) Primary production: The biosphere and man. Hum Eco 1: 357-369
146 Williams AG, Coleman GC (1985) Hemicellulose degrading enzymes in rumen ciliate protozoa. Curr Microbiol 12: 85-90
147 Williams AG, Ellis AB, Coleman GS (1986) Subcellular distribution of polysaccharide deplolymerase and glycosidase hydrolase

enzymes in rumen ciliate protozoa. Curr Microbiol 13: 139-147
148 Williams AG, Strackan NH (1984) The distribution of polysaccharide degrading enzymes in the bovine rumen digesta. Curr Microbiol 10: 215-220
149 Williams PP, Dinussen WE (1973) Ruminal volatile fatty acids concentrations and weight gains of calves reared with and without ruminal ciliated protozoa. J Anim Sci 36: 588-591
150 Wolin MJ (1976) Interaction between $H_2$-producing and methane producing species. In: Schelegel HG, Gottschalk G, Pfennig N (eds) Microbial production and utilization of gases. Goltze KG, Göttingen, p141-150
151 Wolin MJ (1979) The rumen fermentation: A model for microbial interactions in anaerobic ecosystems. Adv Microb Ecol 3: 49-77
152 Wolin MJ (1981) Fermentation in the rumen and human large intestine. Science 213: 1463-1468
153 Wolin MJ, Miller TL (1983) Interactions of microbial populations in cellulose fermentation. Fed Proc 42: 109-113
154 Wood TM, McGrae J (1977) The mechanism of cellulase action with particular reference to the $C_1$ component. In: Ghose TK (ed) Bioconversion of cellulosic substances into energy, chemicals and microbial protein. Indian Institute of Technology, New Delhi, p 111-141
155 Young JC, McCarty PL (1967) The anaerobic filter for waste treatment. In: Proc 22nd purdue Industrial waste Conference, May 1967. Ann Arbor Science publishers Inc, Ann Arbor MI, p 559-574
156 Zeikus JG (1980) Chemical and fuel production by anaerobic bacteria. Ann Rev Microbiol 24: 423-464
157 Zeikus JG (1980) Microbial populations in digesters. In: Stafford DA, Wheatley BI, Hughes DE (eds) Anaerobic digestion. Applied Science publishers Ltd, London, p 61-98
158 Zimmerman PR, Greenberg JP, Wandiga SO, Crutzen PJ (1982) Termites: A potentially large source of atmospheric methane, carbondioxide and molecular hydrogen. Science 218: 563-565
159 Zinder SH (1984) Microbiology of anaerobic conversion of organic wastes to methane: recent developments. ASM News 50: 294-298

# CONTINUOUS CULTIVATION OF RUMEN MICROORGANISMS, A SYSTEM WITH POSSIBLE APPLICATION TO THE ANAEROBIC DEGRADATION OF LIGNOCELLULOSIC WASTE MATERIALS

Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986)
Appl Microbiol Biotechnol 25: 155-162

## SUMMARY

An in vitro continuous fermentation device is described which allows the maintenance of a mixed rumen microbial population under conditions similar to those in the rumen. The differences in flow rates of solids and liquids found in the rumen were established in vitro by means of a simple filter construction. A grass-grain mixture was used as a solid growth substrate. During a test period of 65 days the 'artificial rumen' fermenter showed stable operation with respect to ciliate numbers, fibre degradation and volatile fatty acids production. Values obtained were comparable to those found in vivo. Optimal fibre degradation and volatile fatty acids production were maintained when hydraulic retention times (HRT) ranges from 11 to 14 h. At these HRT-values ciliate numbers were maintained at about 8.5 x $10^4$ cells per ml. Ciliate numbers declined drastically at HRT-values above 14 h. A fermenter inoculated with a small volume of rumen fluid (1 : 100, v/v) reached normal protozoal numbers, fibre degradation and volatile fatty acids productions after a start up period of only 8 to 10 days. The possible application of rumen microorganisms for an efficient degradation of lignocellulosic waste material in an artificial rumen digester is discussed.

## INTRODUCTION

Anaerobic waste water treatment has made great progress recently by the development of several advanced high-rate treatment systems such as the anaerobic filter, the upflow anaerobic sludge blanket and the attached film expanded bed reactor (van den Berg 1984). In contrast, anaerobic conversion of solid organic wastes has not been very efficient in terms of volumetric loading capacities of reactors, solid retention times (SRT) and extent of digestion (Hobson et al. 1980; Weland and Cheremisinoff 1975). For this reason application of anaerobic degradation to organic solid materials has been limited mainly to animal wastes and secondary waste water sludge. The low conversion rates are probably due to a low rate of hydrolysis as a limiting step. Therefore, recent research was focussed on the improvement of hydro-

lysis by physical, chemical or biological pretreatments of the waste materials (Millet et al. 1975; Han et al. 1975), by increasing microbial biomass retention (Callender and Barford 1983) or by screening for more active hydrolytic organisms (Hobson et al. 1984). However, in nature several anaerobic microbial ecosystems are highly active in the conversion of lignocellulosic materials. Typical examples are the digestive tract of termites and the rumen of ruminants.

The controlled environmental conditions in the rumen favour the growth of an extensive and complex microbial population, which mainly consists of bacteria and ciliate protozoa (Hungate 1966). The fermentation pattern of plant polymers by the rumen microbial community is comparable to that observed in anaerobic digesters, but acetate production from volatile fatty acids (VFA) and acetoclastic methane formation are much less important in the rumen. Consequently, acetate, propionate, butyrate and methane originating from $H_2/CO_2$ are the main end products of rumen fermentation.

Application of rumen microorganisms in a rumen-like fermentation system might strongly enhance the industrial fermentation of lignocellulosic waste materials. However, long-term cultivation of rumen microorganisms, including rumen ciliates, in an 'artificial rumen' digester with a simple construction and operation is a prerequisite for such an application. The present paper describes a simple long-term continuous cultivation system for rumen microorganisms. An evaluation of the effects of hydraulic retention time and inoculum volume on fermenter performance, using grass as a substrate, is presented.

## MATERIALS AND METHODS

### Fermentation system

A continuous in vitro fermentation system with differential removal rates of solid and liquid fermenter contents, adapted from the system described by Hoover et al (1976), was used. A schematic diagram of the fermenter is shown in Fig 1.

The culture was kept in a fermenter (a) which consisted of a 3

litre double wall glass vessel (working volume 1.5 litre) and was kept at 39°C by means of a temperature bath circulator (b). Buffer solution (c), according to Rufener et al. (1963) was added continuously to the fermenter at a rate adjustable by peristaltic pump d. Liquid fermentation medium was continuously removed through a filter unit (e) by peristallic pump f and collected in a calibrated vessel. The filter unit consisted of a cylinder (6 cm high, 4 cm diameter) of stainless steel wire gauze (pore size 0.3 mm), which was connected to a glass tube by means of a perspex disc at the top of the cylinder. The cylinder was completely wrapped in a single layer of nylon gauze (30 µm pore size), which was fitted to the glass tube by means of a rubber o-ring. This construction revealed a total filter area of about 90 cm² (25% pore area). The fermenter contents were mixed every 30 minutes for a period of 45 seconds by means of a laboratory rotary shaker (g), which contained maximally 4 fermentation vessels. Immediately

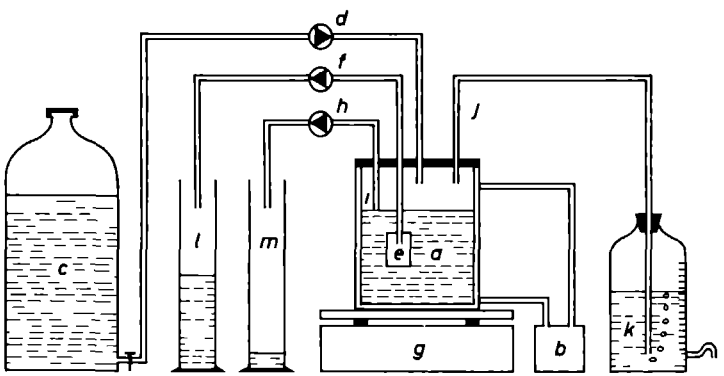



Fig. 1. Schematic diagram of the artificial rumen fermenter.
a, 3 litre cylindrical glass vessel; b, temperature bath circulator; c, buffer vessel; d, f, peristaltic pumps; h, peristaltic pump connected to a timer; e, cylindrical filter; g, laboratory rotary shaker, containing up to 4 fermenter vessels; i, outlet tube for homogeneous fermenter contents; j, outlet for biogas; k, calibrated mariotte bottle; l, filter effluent reservoir; m, homogeneous effluent reservoir.

after mixing pump h was switched on automatically to remove homogeneous fermenter contents through an overflow tube (i) until a fermenter volume of 1.5 litre was reached. The gas outlet (j) of the fermenter was connected to a 10-litre calibrated mariotte flask (k) containing acidified (0.02% HCl) tap-water.

## Experimental conditions

Inoculation. All experiments, except one, were started by inoculating the fermenters with 500 ml strained rumen fluid obtained from a fistulated sheep. After inoculation the fermenters were filled to a working volume of 1.5 litre with buffer solution. In one experiment only 15 ml inoculum was used. In this case, before inoculation, the fermenters were filled with buffer solution (1.5 litre) and flushed with $N_2/CO_2$ (80/20, v/v) for 30 minutes in order to create anaerobic conditions.

Substrate. Immediately after inoculation 70 g dry weight (dw) of a grass-grain mixture (4:1, w/w) according to Hoover et al (1976) were added as a solid growth substrate. On following days the substrate was added in two equal portions daily at loading rates of 20 or 35 g dw per litre fermenter volume per day (g dw/l FV.d).
The grass-grain mixture as determined by the method of Goering and van Soest (1970) contained (w/w): 19% cellulose (C), 28% hemicellulose (HC), 4% lignin (L), 2% ash, and 47% natural detergent soluble organic matter.

Solids (SRT) and hydraulic retention time (HRT). Flow rates of solids and liquids were adjusted to desired values by means of adjustable peristaltic pumps d (buffer input) and f (filtered effluent). HRT was adjusted by pump d, whereas retention of the feed was determined by the difference between buffer and filter flows:

$$\text{SRT (h)} = \frac{FV}{dV-fV} \times 24 \text{ h},$$

in which dV and fV are the daily buffer input volume and filter effluent volume, respectively.

## Digestion

Digestion of the substrate was estimated during steady state, which was reached within 5-7 days after inoculation. Digestion was calculated as follows:

$$\text{digestion (\%)} = \frac{(Fo + S) - (Fx + E)}{S} \times 100\%, \text{ in which}$$

Fo and Fx: fibre contents in fermenters at T = o and T = x, respectively. x is usually 3 days.

E: fibre contents in total fermenter effluents (filtrate and homogeneous fermenter effluent), collected during time x.

S: fibre contents of the substrate added during time x.

Fo was generally almost similar to Fx as should be expected during steady-state performance.

In order to allow sampling at T = o without a decrease of the working volume of the fermenters below 1.5 litre, pump h was switched off the day before sampling, resulting in an increase of the fermenter contents. After removal of 4 homogeneous 50-ml samples at T = o, the fermenter volume was adjusted to 1.5 litre by pump h. During the period in which digestion was determined, filtered and homogeneous effluents were combined and stored at 4°C. At the end of the digestion period (T = x) again 4 homogeneous samples were taken from the fermenter contents and from the combined effluents. All samples were stored at -20°C until fibre analyses were performed.

## Analytical methods

Fibre analyses. Neutral detergent fibre (NDF), acid detergent fibre (ADF), C, HC and L contents of the samples were determined according to the method of Goering and van Soest (1970). NDF and ADF analyses of the 50 ml samples were performed with an equal volume of double strength detergent solutions. The same analyses were carried out with 0.5 g dry substrate suspended in 50 ml demineralized water. Each ana-

lysis was performed in duplo. After determination of the dry weight all fibre residues were ashed (550°C, 3 h) to allow the results to be expressed on an ash-free basis.

Volatile fatty acids (VFA). 5-ml Samples for determination of pH and VFA were taken from the combined 24-hour effluent every other day. After measurement of pH, the samples were stored at -20°C until VFA analysis. Samples of 1 ml were mixed with 0.1 ml 100 mM isobutyric acid as an internal standard. Subsequently 0.25 ml 20% ortho-phosphoric acid was added and after thoroughly mixing the samples were allowed to stand for 30 minutes. After removal of the solids by centrifugation the supernatant was used directly for gaschromatographic analysis of individual fatty acids by use of a Pye Unicam model GCD gaschromatograph equiped with a flame ionization detector. 0.1-0.2 µl samples were injected into a glass column filled with 10% SP1200/1% $H_3PO_4$ on 80/100 chromosorb WAW (6 ft x 2 mm ID) at a temperature of 130°C. Nitrogen was used as a carrier gas at a flow of 40 ml/min. Injection and detection temperatures were 170°C and 175°C, respectively.

Protozoal numbers. Protozoal numbers were determined every other day in a 3.6 ml sample removed from the fermenters 4 h after the first of the daily substrate additions. The samples were fixed in 0.4 ml 37% formaldehyde and stored at room temperature. Protozoal counts were made by means of a Reichert MeF inverted microscope with the use of a calibrated 4 ml cuvette containing a 20-fold diluted sample.

Biogas. Daily biogas productions were monitored by means of a calibrated mariotte flask containing acidified (0.02% HCl) tap water.

## RESULTS

### Long-term operation

In order to establish the stability of the continuous in vitro rumen fermentation system, a long term experiment was performed over a period of 65 days. HRT and SRT were adjusted to 12 and 60 h, respectively. Actual performance data for HRT and SRT expressed as means of daily measurements (± SD) were 12.2 ± 0.6 h and 60.6 ± 3.9 h.

During the entire period no filter change or filter regeneration was needed. Day to day variations in protozoal numbers during the 65 days fermentation period are shown in Fig. 2. Immediately after inoculation the number of protozoa declined, reaching a relatively constant level of about $65 \times 10^3$ cells/ml after 3 days of incubation.

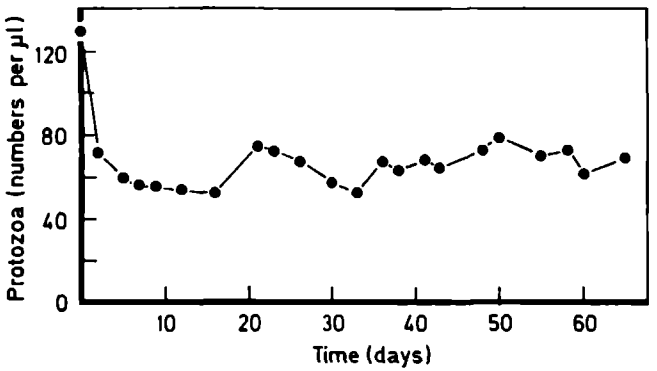



Fig. 2. Variations of protozoal numbers in the fermenter contents during long-term operation.

For establishment of the relative numbers of the different ciliate species, the ciliates were subdivided into 4 groups; the *Diplodinium* group (*Eudiplodinium maggii* and *Diplodinium dentatum*), the *Epidinium* group (*Epidinium ecaudatum*), the *Entodinium* group (*Entodinium simplex*, *E. longinucleatum*, *E. caudatum*), and holotrichs (*Isotricha prostoma* and *Dasytricha ruminantium*). The relative number of the ciliates of these groups during different stages of the test period are shown in Table 1. The ciliates in the inoculum belonged mainly to the holotrichs and to the *Entodinium* group. During the first 8 days of the test period drastic changes in the relative numbers of the different groups were observed: the relative numbers of the *Diplodinium* and *Epidinium* groups increased, whereas the numbers of the *Entodinium* group were strongly lowered and the holotrichs were completely eliminated. The relative numbers of the various groups remained rather stable during the rest of the test period. The changes of protozoal species observed in the transition of the inoculum to continuous cul-

TABLE 1. Frequency (% of total) of different ciliate groups during long-term incubation in a continuous culture

| Group | Inoculum | Day 10 | Day 10-65 (mean ±SD[a]) |
|---|---|---|---|
| *Diplodinium* | 8 | 52 | 50 ± 4 |
| *Epidinium* | 3 | 25 | 26 ± 5 |
| *Entodinium* | 71 | 23 | 24 ± 5 |
| Holotrichs | 18 | 0 | 0 |

[a]Standard deviation (n = 18)

ture are probably due to differences in substrates used, or the fermentation conditions applied, but the fast decrease in the numbers of the *Entodinium* group during the first days of incubation might also be a result of the filter pore size, which is not small enough to retain these ciliates.

The day to day variation in VFA concentration in the filter effluent is shown in Fig 3. The mean (± SD) production (concentration x dilution rate) of total VFA during the test period was 155 ± 13 mmoles/l FV.d. Concentrations of individual VFA were rather constant throughout the test period, and amounted to mean molar percentages of 67%, 21% and 12% for acetic, propionic and butyric acid, respectively. Mean fermenter pH was 6.1 ± 0.2 (SD). The molar percentages of individual VFA are in agreement with in vitro values reported by Erfle et al (1982) at the same pH-value. These authors reported that the molar percentages of individual VFA were greatly affected by fermenter pH. The mean biogas production during the incubation was 4.3 ± 0.5 1/1 FV.d.

Digestion of the substrates was determined twice; directly after equilibration (day 7-10) and from day 40 to 43 (Table 2). NDF digestion was almost the same in both periods, but degradation of cellulose was slightly higher and degradation of hemicellulose was lower in the second period. Such differences may be a consequence of changes in microbial composition between the test periods.

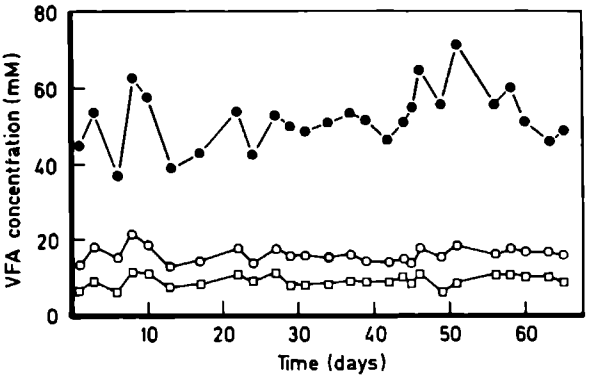



Fig. 3. Concentrations of acetate (●), propionate (o) and butyrate (□) in the filtered fermenter effluent during long-term operation.

TABLE 2. Fibre degradation (% d.w.) during long-term incubation in continuous culture

| Fibre fraction | Period 1 day 7-10 | Period 2 day 40-43 |
|---|---|---|
| NDF | 70 | 67 |
| ADF | 63 | 68 |
| Cellulose | 69 | 73 |
| Hemicellulose | 83 | 64 |
| Lignin | 42 | 53 |

**Effect of HRT on fermenter performance**

The effect of variation of HRT on protozoal numbers and fibre degradation was studied in six short-term experiments. All experiments were performed over a period of 14 days, with SRT at 60 h and substrate loads of 35 g dw/l FV•d, whereas HRT was varied. The effect of

HRT on NDF degradation is shown in Fig 4. The increase of HRT from 11 to 25 h resulted in a decrease of NDF digestion from 66 to 34%. Comparable results were obtained by Crawford et al (1980) with HRT ranging from 6.7 to 14.3 h. Czerkawski and Breckenridge (1977) however reported almost no change of dry matter degradation, with values varying from 60 to 66%, when HRT was increased from 25 to 75 h.

Table 3 summarizes the effect of HRT variation on fermentation products and fermenter pH during steady-state (day 7 - 14). The increase of VFA production observed at higher dilution rate is in agreement with the improved digestion of the substrate. The molar percentages of the individual VFA did not vary markedly among different incubations. The pH in the fermenters decreased with increasing HRT as a result of the combined effects of a decreased buffer input rate and a slower VFA removal.

The variation in HRT had a drastic effect on protozoal numbers in the fermenters (Fig 5). At a HRT of 25 h the protozoal numbers were very low ($2x10^3$ cells/ml). This may be an effect of the low pH observed during the incubation at this HRT (Table 3). When the HRT was

TABLE 3. Effect of HRT on VFA production and fermenter pH. All values are expressed as means ± SD (n = 4) of measurements obtained during steady-state (day 7 - 14)

| HRT (h) | VFA production (mmol/L.FV.d) | | | pH |
|---|---|---|---|---|
| | Acetate | Propionate | Butyrate | |
| 11 | 82.0 ± 5.3 | 28.2 ± 1.2 | 15.9 ± 1.1 | 6.28 |
| 12 | 93.5 ± 23.6 | 29.4 ± 8.1 | 16.3 ± 6.1 | 6.22 |
| 14 | 81.3 ± 13.3 | 24.6 ± 1.0 | 14.1 ± 1.8 | 6.06 |
| 16 | 75.0 ± 6.7 | 27.0 ± 1.3 | 11.4 ± 1.1 | 5.85 |
| 18.5 | 56.5 ± 6.5 | 20.5 ± 1.8 | 13.8 ± 0.7 | 5.95 |
| 25 | 41.7 ± 11.7 | 16.2 ± 2.7 | 10.8 ± 1.7 | 5.61 |

decreased, protozoal numbers increased markedly and reached a constant level of approximately $85x10^3$ cells/ml at a HRT between 11 and 14 h. Crawford et al (1980) and Czerkawski and Breckenridge (1977) found no effect of HRT on protozoal numbers in vitro. These authors reported mean protozoal numbers of about $10^4$ cells/ml and $38x10^3$ cells/ml when HRT was varied between 6.7 and 14.3 h, and between 25 and 75 h, respectively.

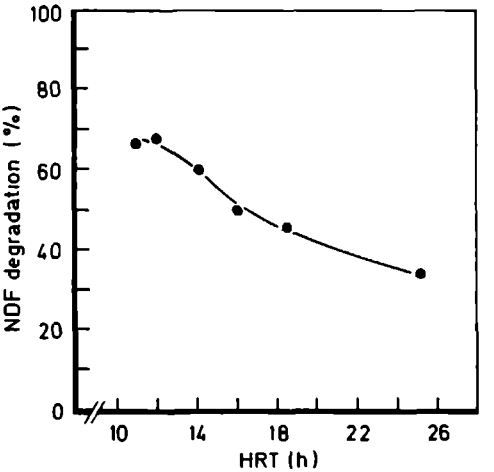



Fig. 4: Effect of variation of HRT on NDF degradation.

**Dilute inoculation**

In order to study the start-up of a reactor after dilute inoculation, a fermenter was inoculated with 15 ml of strained rumen fluid (1:100, v/v), and measurements were made over a period of 20 days. SRT was maintained at 190 h in order to minimize washout of protozoa and fibre-associated bacteria. HRT was maintained at 12 h, and a grass-grain mixture was added at a load of 20 g dw/l FV.d.

Fig 6 gives the course of ciliate numbers in the fermenter as a function of incubation time. The ciliate numbers in the reactor were $2.4x10^3$ cells/ml directly after inoculation and reached a steady state level of about $90x10^3$ cells/ml within 10 days. After inoculation of the fermenter, the frequency of different protozoal groups changed rapidly (Table 4). The relative number of the different groups after

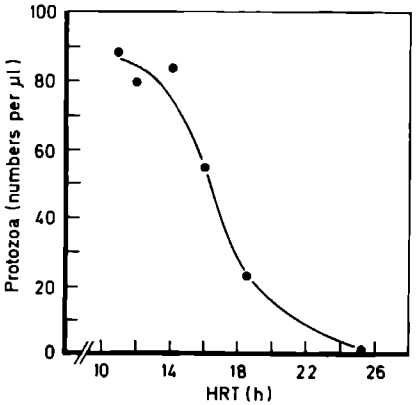



Fig. 5: Effect of variation of HRT on protozoal numbers in the fermenter contents.

stabilization was comparable to those observed during the long-term incubation (Table 1), except that the frequency of the *Entodinium* group was somewhat lower. Although the frequency of the holotrichs decreased drastically during the incubation, their absolute number increased almost tenfold. This is in contrast with the observation in previous experiments, where holotrichs completely disappeared after stabilization. The maintenance of holotrichs in this experiment is probably a consequence of the relatively long SRT applied.

The production of VFA also reached steady-state levels within 8 to 10 days after incubation. The mean (± SD) productions of acetic, propionic and butyric acid between day 10 and day 20 of incubation were 75.5 ± 15.4, 18.2 ± 3.3 and 15.1 ± 1.7 mmoles/d, respectively. These productions were somewhat lower as compared to the long-term incubations as a result of the lower loading rate applied. Fibre degradation was determined between day 8 and 19 after inoculation (Table 5). The extent of fibre degradation, except for lignin, was comparable to the degradation observed during long-term operation at a loading rate of 35 g dw/l.d. (Table 2). In spite of the lower loading rate and longer SRT, the degradation of cellulose and hemicellulose were only slightly improved, whereas lignin degradation was negligible low. The cellulose and hemicellulose remaining after advanced digestions may be more resistant to enzymatic hydrolysis.

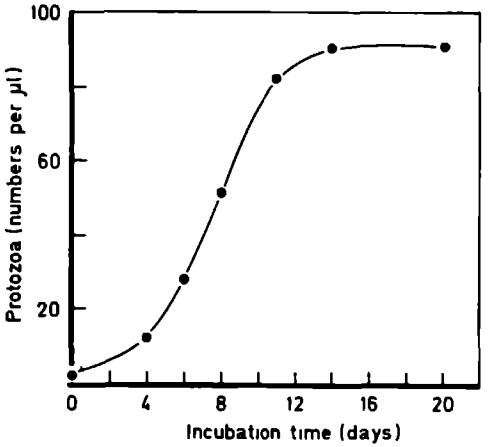



Fig. 6: Growth of ciliates in the artificial rumen fermenter after inoculation with 15 ml (1:100, v/v) rumen fluid

TABLE 4. Frequency (% of total) of different ciliate groups in continuous culture at day 1 (inoculum) and between day 10 and day 20 after inoculation with 15 ml (1:100, v/v) rumen fluid

| | day 1 | day 10-20 |
|---|---|---|
| *Diplodinium* | 11 | 58 |
| *Epidinium* | 3 | 27 |
| *Entodinium* | 74 | 12 |
| Holotrichs | 12 | 3 |

## DISCUSSION

The rumen fermentation system is one of the most active anaerobic ecosystems involved in the degradation of vegetable material. For this reason application of rumen fermentation processes to the degradation of lignocellulosic waste materials in an industrial scale anaerobic

TABLE 5. Fibre degradation (% d.w.) in continuous culture between day 8 and 19 after inoculation with 15 ml (1:100, v/v) rumen fluid

| Fibre fraction | Degradation (%) |
|---|---|
| NDF | 74 |
| ADF | 59 |
| Cellulose | 71 |
| Hemicellulose | 88 |
| Lignin | 3 |

digester could be of great importance provided that:

1. Microbial biomass retention and growth yields during the in vitro fermentation are similar to those found in vivo.
2. Digester performance is stable over extended periods of time.
3. Replacement of the natural substrates like grass by lignocellulosic wastes results in similar activities of the rumen microbial population.

Therefore it is of great importance to develop a continuous fermentation system which closely approximates in vivo conditions. A major characteristic of the rumen is that retention times of microbial biomass and solid substrate are significantly longer compared to the retention time of the fluid (Weller and Pelgrim, 1974). It has been demonstrated that most of the rumen microorganisms, comprising bacteria, protozoa and fungi are associated with the food particles, either by attachment or by sequestration (Akin and Barton 1983). In this way an efficient removal of acid fermentation products together with a prolonged retention of rumen microorganisms is ensured.

A number of continuous culture devices have been reported with the primary goal to study rumen microbial fermentation (Rufener et al 1963; Slyter et al 1964; Abe and Kumeno 1973; Weller and Pelgrim 1974; Hoover et al 1976; Abe and Kurihara 1984). In continuous culture an efficient end product removal and biomass retention have been accomplished by means of dialysis (Abe and Kumeno 1973), sequestration

(Weller and Pelqrim 1974; Czerkawski and Breckenridge 1977; Abe and Kurihara 1984), or by filtering techniques (Hoover et al 1976). Because dialysis and sequestration techniques are probably difficult to realize in full scale digester design, a filtration technique was used in this study. The filtration technique showed stable performance during long-term operation without regeneration or changing of filters. This is an improvement with respect to the filtering technique described by Hoover et al (1976), who reported that filters needed to be changed every other day in order to keep filter flows constant.

The results of the short-term incubations conducted at different HRT values indicated that optimum fermenter performance was maintained when HRT ranged from 11 h to 14 h. These values approximate HRT values that have been reported for the in vivo situation (Hungate 1966). The decrease in fibre degradation and protozoal numbers observed at decreased fluid flow rates may be a result of increased end product concentrations and a lower fermenter pH under these conditions. It has been demonstrated in numerous studies that a low pH has a negative effect on rumen fermentation in vivo and in vitro (References in Erfle et al 1982). Since variations of fluid flow rates affect both pH and acid product removal rates directly, it was not possible to evaluate the independent effects of pH and VFA concentrations in this study. By using pH-controlled continuous cultures, Hoover et al (1984) found that fibre degradation was more closely related to pH-variations than to dilution rates. These results, together with our observation that VFA concentrations in the fermenters were relatively constant at different HRT values, suggests that a short HRT is necessary in order to prevent acidification of the fermenter contents. Together with the need for a relatively high fluid removal rate, SRT must be long enough to prevent washout of rumen microorganisms. This means that the differential removal rates for solids and liquids are of utmost importance in order to maintain the efficiency of the fermentation process.

The results of the 65-days fermentation period clearly demonstrate that, after an equilibration period of approximately 5-7 days, steady-state conditions could be maintained with respect to ciliate numbers, fibre degradation and fermentation product formation. Except for ciliate numbers, which were somewhat lower, all other parameters measured were comparable to values reported for the in vivo situation

(Weller and Pelgrim 1974; Hungate 1966).

Lignin degradation during the first and second digestion period were measured to be 42 and 53%, respectively. Contrary to previous reports (Zeikus et al 1982) in which it was stated that lignin is not degradable in the absence of oxygen, Benner et al (1984) presented evidence for lignin degradation by sediment microflora under anaerobic conditions. However, this process proceeded very slowly. In our experiments SRT was only 60 h and therefore it is unlikely that the loss of lignin measured at this retention time was due to anaerobic degradation. The apparent digestion of lignin is probably due to the solubilization of low molecular lignin through the action of rumen microorganisms on forage, as was reported before (Gaillard and Richards 1975; Fahay and Jung 1983). This is in agreement with our observation that oatstraw lignin becomes soluble during anaerobic incubation with *Trichoderma* cellulase (Gijzen et al unpublished results). Therefore the apparent degradation of lignin observed in the rumen or during in vitro rumen fermentation is probably a secondary effect resulting from the fermentation of other cell wall polymers. Consequently lignin should not be used as a marker during in vivo digestion studies.

A limitation for the application of rumen microorganisms to the industrial fermentation of lignocellulosic waste materials could be caused by the relatively small inoculum volume which is available for start up of a digester. As a consequence, a relatively small number of rumen microorganisms should be able to grow out to normal numbers in a digester. Growth of rumen microorganisms in the rumen of meroxenic lambs receiving a small inoculum of rumen fluid is known to be a fast process (Fonty et al 1983). Our results show that also in vitro high numbers of protozoa were reached within 8-10 days after inoculation with rumen fluid at a 100-fold dilution. The same is probably true for bacterial numbers, since Coleman (1975) demonstrated that growth of ciliates is related to bacterial numbers in protozoal cultures. Thus a small inoculum volume results into normal numbers of microorganisms and consequently in normal fermentation characteristics after a relatively short adaptation period.

An important question that remains to be answered is whether replacement of the natural substrate grass by lignocellulosic waste materials is possible without significantly decreasing fermenter per-

formance. Since many types of organic wastes are already being used as an animal feedstuff, especially for ruminants, the anaerobic degradation of these materials by rumen microorganisms in a digester should be possible too.

The reactor performance data described in this study provide a basis for further studies with respect to the fermentation of lignocellulosic waste materials. The predominant end products of the rumen fermentation process are VFA and to a lesser extent biogas. Since VFA concentrations are too low for an economically feasible separation of these acids as a source of bulk production, it seems more attractive to convert these products into biogas. The coupling of the artificial rumen fermenter to a methane reactor and the utilization of lignocellulosic waste materials will be reported in a next series of papers.

## REFERENCES


Abe M, Kumeno F (1973) In vitro simulation of rumen fermentation: Apparatus and effects of dilution rate and continuous dialysis on fermentation and protozoal population. J Anim Sci 36:941-949

Abe M, Kurihara Y (1984) Long-term cultivation of certain rumen protozoa in a continuous fermentation system supplemented with sponge materials. J Appl Bacteriol 56:201-213

Akin DE, Barton FE (1983) rumen microbial attachment and degradation of plant cell walls. Fed. Proc. 42: 114 - 121

Benner R, Maccubbin AE, Hodson RE (1984) Anaerobic biodegradation of the lignin and polysaccharide components of lignocellulose and synthetic lignin by sediment microflora. Appl Environm Microbiol 47:998-1004

Callander IJ, Barford JP (1983) Recent advances in anaerobic digestion technology. Process Biochemistry 18:24-30

Coleman GS (1975) The interrelations between rumen ciliate protozoa and bacteria. In: McDonald IW, Warner ACI (eds) Digestion and metabolism in the ruminant. Armidale, New South Wales, Australia: University of New England Publishing unit, p. 149-164

Crawford RJ Jr, Hoover WH, Knowlton PH (1980) Effects of solids and liquids flows on fermentation in continuous culture. I. Dry matter and fibre digestion, VFA production and protozoa numbers. J Anim Sci 51:975-986

Czerkawski JW, Breckenridge G (1977) Design and development of a long-term rumen simulation technique (Rusitec). Br J Nutr 38:371-385

Erfle JD, Boila RJ, Teather RM, Mahadevan S, Sauer FD (1982) Effect of pH on fermentation characteristics and protein degradation by rumen microorganisms in vitro. J Dairy Sci 65:1457-1464

Fahay GC Jr, Jung HG (1983) Lignin as a marker in digestion studies: A review. J Anim Sci 57:220-225
Fonty G, Govet Ph, Jouany JP, Senaud J (1983) Ecological factors determining establishment of cellulolytic bacteria and protozoa in the rumens of meroxenic lambs. J Gen Microbiol 129:213-223
Gaillard BDE, Richards GN (1975) Presence of soluble lignin-carbohydrate complexes in the bovine rumen. Carbohydr Res 42:135-145
Goering HK, van Soest PJ (1970) Forage fiber analysis. USDA, ARS Agricultural Handbook No. 379
Han YW, Pence JW, Anderson PW (1975) Dioxane treatment to improve microbial digestibility of cellulosic fibers. Appl Microbiol 29: 708-709
Hobson PN, Bousfield S, Summers R (1974) Anaerobic digestion of organic matter. CRC Crit Rev Environm Control 4:131-191
Hobson PN, Reid GW, Sharma VK (1984) Anaerobic conversion of agricultural wastes to chemicals or gases. In· Ferrero GL, Ferranti MD, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste. Elsevier applied science publishers, London, New York, p. 369-381
Hoover WH, Crooker BA, Sniffen CJ (1976) Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen content. J Anim Sci 93: 528-535
Hoover WH, Kincaid CR, Varga GA, Thayne WV, Junkins LL Jr (1984) Effects of solids and liquid flows on fermentation in continuous cultures. IV pH and dilution rate. J Anim Sci 58:692-700
Hungate RE (1966) The rumen and its microbes. Academic Press, New York
Millet MA, Baker AJ, Satter LD (1975) Pretreatments to enhance chemical, enzymic and microbiological attack of cellulosic materials. Biotechnol Bioeng Symp 5:193-219
Rufener WH Jr, Nelson WO, Wolin MJ (1963) Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11:196-201
Slyter LL, Nelson WO, Wolin MJ (1964) Modifications of a device for maintenance of the rumen microbial population in continuous culture. Appl Microbiol 12:374-377
Van den Berg L (1984) Developments in methanogenesis from industrial waste water. Can J Microbiol 30:975-990
Weland AF, Cheremisinoff PN (1975) Anaerobic digestion of sludge II. Water Sewage Works 122:45-47
Weller RA, Pelgrim AF (1974) Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Br J Nutr 32:341-352
Zeikus JG, Wellstein AL, Kirk TK (1982) Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. FEMS Microbiol Lett 15:193-197

# CONTRIBUTION OF RUMEN PROTOZOA TO FIBRE DEGRADATION AND CELLULASE ACTIVITY IN VITRO

Gijzen HJ, Lubberding HJ, Gerhardus M, Vogels GD,
(submitted for publication)

## SUMMARY

The contribution of ciliates to rumen fermentation was estimated by determination of overall fibre degradation and cellulase activities in faunated and defaunated 'artificial rumen' cultures. Experiments performed at loading rates of 22.5 and 35 $g.l^{-1}.d^{-1}$ of a grass-grain substrate revealed that fibre degradation was significantly lower in the absence of ciliates only at the high loading rate. This effect of defaunation was smaller at dilution rates below 1.7 fermenter volume turnovers per day. Bacterial numbers were higher in all experiments after removal of ciliates. Fractionation studies demonstrated that ciliates accounted for 19-28% of the total cellulase activity in faunated cultures fed on filter paper cellulose.

## INTRODUCTION

Fibre degradation in the rumen is carried out by a mixed population of various kinds of bacteria, ciliate protozoa and phycomycete fungi. Although much is known about the predominant hydrolytic bacteria which have been studied in pure culture, the role of rumen ciliates in the degradation of plant cell wall polymers is still controversial.

A wide range of plant cell wall degrading enzymes have been detected in various species of rumen ciliates (Orpin, 1984; Coleman, 1986) and electron microscopic studies have revealed that they were involved in fibre degradation (Akin and Amos, 1979). Ciliate protozoa are present in large numbers and their biomass approximates that of the bacteria (Hungate, 1966). For this reason ciliates were considered to be essential to the ruminant, but many in vivo studies have demonstrated that ruminants can survive without a protozoal population present. However, fibre degradation and animal weight gain were usually lower in the absence of ciliates (Abou Akkada and El-Shazly, 1964; Kurihara et al, 1978; Jouany and Senaud, 1979) although no effect or a positive effect of defaunation has been reported as well (Luther et al, 1966; Eadie and Gill, 1971; Brid and Leng, 1978). The contradictory results might be attributable to in vivo conditions which are too complex and varia-

ble.

The present study was conducted to establish the effect of defaunation on fibre degradation in vitro by means of an 'artificial rumen' fermenter (Gijzen et al, 1986), in which conditions are more reproducible. The role of ciliates was established at different conditions of loading rate and substrates applied. In order to quantify the involvement of ciliates in fibre digestion, cellulase activity was determined in various fractions of faunated and defaunated cultures fed on cellulose.

## MATERIALS AND METHODS

### Fermenter design and operation

In vitro fermentations were carried out in 3 L volume continuous cultures at 39°C with differential removal rates of solids and liquids, as was described previously (Gijzen et al, 1986). A fistulated sheep was the source of strained rumen content (250 ml) for fermenter inoculation. The solid substrates consisted of a grass-grain mixture according to Hoover et al (1976) or of a mixture of filterpaper cellulose (Whatman, grade 91) and ground alfalfa (van Heeswijk, Veghel, the Netherlands). The substrates were added once daily at desired loading rates, except at the day of inoculation, when twice the normal loading rate was fed. The chemical composition of the substrates is shown in Table 1. Mineral buffer solution according to Rufener (1963) was used as a fermentation medium. During the experiments with the grass-grain substrate. If cellulose was used as a predominant fermenter feed, the fermentation medium was supplemented with 28 mmol.$l^{-1}$ $NH_4Cl$ (loading rate of 13 g cellulose.$l^{-1}$.$d^{-1}$) or $NH_4HCO_3$ (loading rates of 15 or 20 g.$l^{-1}$.$d^{-1}$) and trace elements (0.2 ml.$l^{-1}$) according to Vishniac and Santer (1957).

Desired retention times of solids (SRT) and liquids were established by adjusting the rate of fermentation medium supply and filtered effluent removal. Homogeneous fermenter contents was removed once daily, shortly before substrate addition. All fermenters were operated at an average working volume of 1.5 l.

Table 1. Composition of the substrates (means ±SD)

| Determination | Grass-grain | alfalfa | FPC[a] |
|---|---|---|---|
| Dry weight (%) | 93.8±0.9 | 92.9±0.8 | 93.9±0.1 |
| Ash | 2.0±0.2 | 7.6±0.4 | 0.2±0.1 |
| NDF | 48.1±1.7 | 47.3±0.3 | nd[b] |
| ADF | 23.1±0.5 | 33.6±0.5 | nd |
| Hemicellulose | 25.0±1.8 | 13.7±0.5 | nd |
| Cellulose | 19.1±0.7 | 25.6±0.3 | 98.5 |
| Lignin | 4.0±0.5 | 8.0±0.8 | nd |
| Cell solubles | 49.9±1.7 | 45.1±0.4 | 1.3 |

[a] Filter paper cellulose

[b] nd = not determined

**Defaunation**

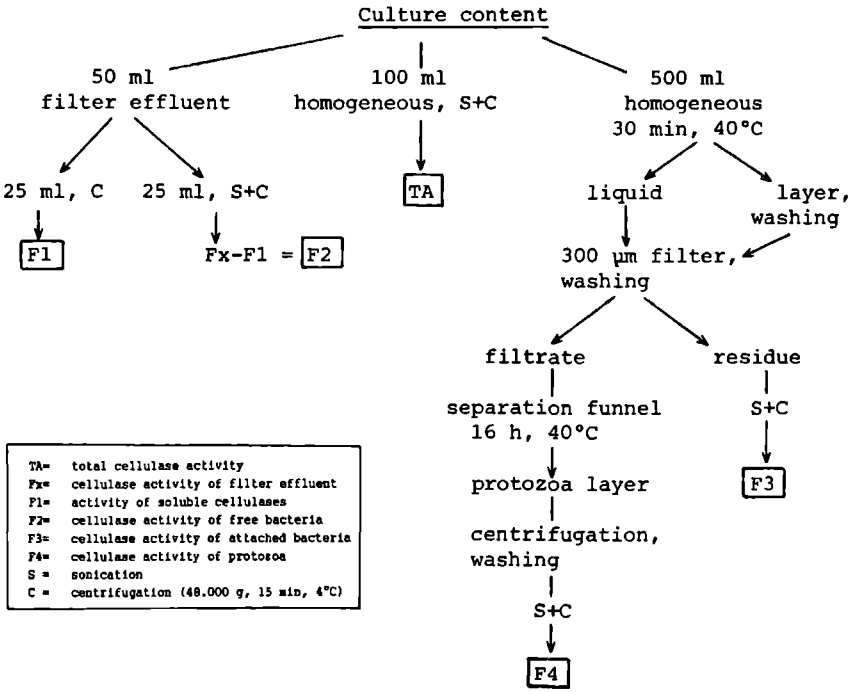
Experiments were started by simultaneous inoculation of two fermenters, which were operated at completely identical conditions. After an acclimatization period of 7 days, one of the fermenters was defaunated by the addition of cycloheximide (0.47 mM). After another acclimatization period of 7 days fermentation products and fibre degradation were determined over periods of 7 and 3 days, respectively. During this period 10-ml samples were withdrawn from the fermenter contents every other day for determination of pH, VFA, and protozoal numbers.

**Cellulase distribution among bacteria and protozoa**

A schematic flow diagram of the separation of bacteria and ciliate protozoa is presented in Fig 1. The fractionation was modified from the procedure described by Williams and Stracken (1984).

Total activity. Total activity was determined on homogeneous culture content (TA).

Figure 1. Schematic diagram of the fractionation of culture content




Activity in filter effluent. Soluble cellulase activity was determined on supernantant obtained after centrifugation (48,000 x g, 10 min, 4°C) of filter effluent (F1). Cellulase activity of free bacteria (F2) was calculated by subtracting F1 from the activity of total filter effluent (Fx).

Activity of attached bacteria. Homogeneous culture content was allowed to stand for 30 min at 40°C, resulting in a floating layer of substrate and a liquid phase. The liquid was removed by suction and filtered. The substrate layer was mixed with 250 ml fermentation medium (40°C) and filtered. Total filter residue was washed once with 250 ml fermentation medium (40°C). Activity of attached bacteria was determined on the filter residue (F3).

Activity of ciliates. Ciliates present in the combined filtrate were allowed to settle down in a seaparation tunnel. The layer of protozoa was centrifuged gently (250 g, 2 min) and cellulase activity

of the obtained pellet was determined (F4).

During the entire procedure ciliates were still viable under the microscope, although their total number decreased. Protozoa were counted in all steps of the fractionation procedure. Corrections were made for the loss of protozoa during the fractionation procedure.

**Analytical methods**

Neutral detergent fibre (NDF), acid detergent fibre (ADF), hemicellulose (HC), cellulose (C) and lignin (L) (Permanganate method) content of substrates and of fermenter samples (50 ml) taken during the 3 days digestion period were analysed in duplo according to Goering and van Soest (1970). The extent of fibre digestion was calculated as described previously (Gijzen et al, 1986). Biogas production was measured daily by means of a 10 l mariotte flask containing acidified tap water (about 0.02% HCl) and methane content was determined according to Hutten et al (1981).

Bacterial numbers were determined during steady-state on 1 ml samples of homogeneous fermenter content. After addition of 1 ml bidest, the samples were mixed vigorously for 5 min and subsequently diluted (100 fold) with fermentation medium. Ciliates and plant fibres were removed by centrifugation (75 g, 10 min) and bacterial numbers in the supernatant were measured by microscopical enumeration. Since it was not possible to release all the bacteria attached to the substrates, bacterial numbers determined in this way should be regarded as a relative measure of the total number. Determination of VFA, protozoal numbers and species distribution, were as described previously (Gijzen et al, 1986).

Cellulase activity was determined on the various fractions derived from the fractionation procedure (Fig 1). Cells, in a volume of 25 ml, were broken by ultrasonic disintegration (Branson B12, Danburg, Connecticut) at 4°C in the presence of glass beads (Ø 4 mm). After five 30-S periods of sonication with 30-S intervals, the mixture was centrifuged at 48,000 g during 15 min. The pellet was resuspended in distilled water and the same procedure was repeated two times. By the three successive sonication steps about 90% of the cellulase activity was extracted from the cells (Lubberding et al, in preparation).

Cellulase activity was determined in the supernatant by measuring the amount of reducing sugars released from carboxymethylcellulose (CMC, 0.5% in 0.1 M Na-acetate buffer, pH 5.5) after 20 min. The content of reducing sugars was determined according to the Nelson and Somogyi procedure (Somogyi, 1952).

## RESULTS

### Effect of defaunation on fibre degradation

Preliminary experiments with respect to various defaunation methods indicated that a temperature shock (48°C, 30 min) was very effective in the elimination of ciliates in mixed cultures of rumen micro-organisms (results not shown). However, it cannot be excluded that this defaunation method also affects bacterial populations and therefore cycloheximide was used in this study as a more specific defaunating agens acting on eucaryotic cells only.

The digestion of a grass-grain mixture in faunated and defaunated continuous cultures was established in two sets of experiments at loading rates of 22.5 and 35 $g.l^{-1}.d^{-1}$. Dilution rate and SRT were adjusted at 1.8-2.2 fermenter volume turnovers per day ($FV.d^{-1}$) and 60 ± 1 h, respectively, for both sets of experiments. Steady-state degradation of cell wall polymers (NDF) was almost similar in faunated and ciliate free cultures operated at a loading rate of 22.5 $g.l^{-1}.d^{-1}$ (Table 2). However, at a loading rate of 35 $g.l^{-1}.d^{-1}$ degradation efficiencies of all fibre fractions except HC were significantly higher ($P<0.01$) in faunated cultures (Fig 2). The differences were most pronounced for cellulose and lignin fibre fractions, whereas the difference in HC digestion was not significant ($P>0.05$).

A similar tendency was observed for fermentation product formation at loading rates of 22.5 and 35 $g.l^{-1}.d^{-1}$ (Table 2). Elimination of ciliates had no effect on fermentation products at a loading rate of 22.5 $g.l^{-1}.d^{-1}$, but at the higher loading rate defaunation resulted in a decreased production of VFA ($P<0.01$) and biogas ($P>0.05$). The individual variations in biogas production were too large to show a

Table 2. Effect of loading rate on steady-state fermentation (means ± SE) in faunated and defaunated continuous cultures fed on a grass-grain mixture

| Determination | loading rate ($g.l^{-1}.d^{-1}$) | | | |
|---|---|---|---|---|
| | 20[a] | | 35[b] | |
| | F[c] | DF[d] | F | DF |
| VFA production ($mmol.l^{-1}.d^{-1}$) | 105±4 | 101±3 | 141±3 | 128±7 |
| molar %, acetate | 66 | 66 | 66 | 67 |
| propionate | 21 | 22 | 21 | 21 |
| butyrate | 13 | 12 | 13 | 12 |
| pH | 6.73±0.05 | 6.77±0.03 | 6.11±0.17 | 6.26±0.16 |
| Methane production ($l.l.^{-1}.d^{-1}$) | 1.6±0.2 | 1.5±0.2 | 2.2±0.3 | 1.8±0.2 |
| Ciliate number ($10^3.ml^{-1}$) | 57±12 | - | 85±20 | - |
| Bacterial number ($10^8 ml^{-1}$) | 36[e] | 75[e] | 39±2 | 89±17 |
| NDF degradation (%) | 73.7±5.3 | 73.0±5.8 | 66.0±3.3 | 56.3±2.7 |

a n = 3
b n = 6
c faunated
d defaunated
e n = 2

significant difference. In contrast to the often reported increase in the proportion of propionate or butyrate by defaunation (Demeyer, 1981; Jouany et al, 1981), no effect of ciliates was observed in this study with respect to the molar proportions of invididual VFA. As is shown in Table 2, the elimination of ciliates resulted in a considerable increase in bacterial numbers at both loading rates.
Although steady-state ciliate numbers were somewhat higher at the high loading rate, population composition was not altered by loading rate.

To establish the effect of dilution rate on NDF digestion in both faunated and defaunated cultures 5 pairs of experiments were performed at dilution rates varying from 1.3 to 2.2 $FV.d^{-1}$. SRT and loading rate were 60 h and 35 $g.l^{-1}.d^{-1}$, respectively, for all experiments.

Previously we demonstrated that low dilution rates had a negative

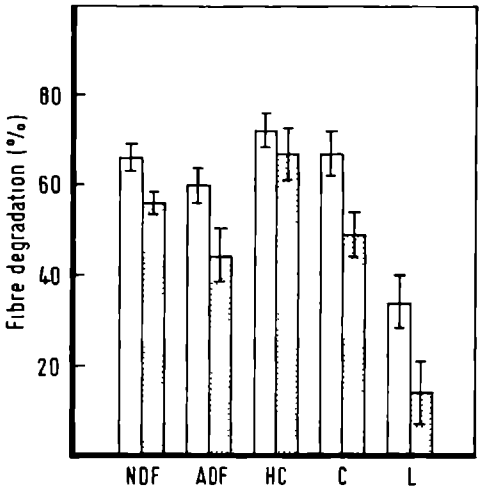



Figure 2. Steady-state fibre degradation in the presence (▭) and absence (▭) of ciliates. (average ± SE, n = 6)

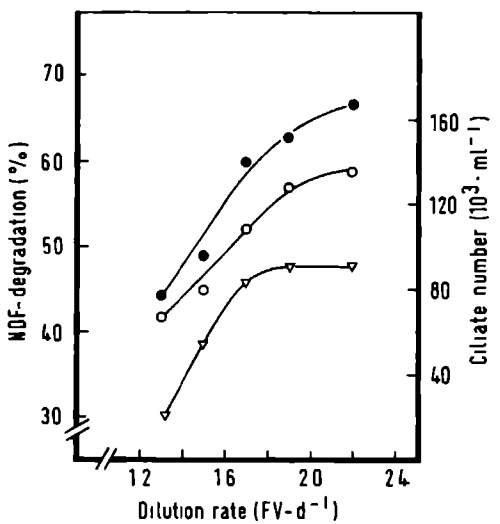



Figure 3. Effect of dilution rate on steady-state ciliate number and NDF degradation in faunated (●) and defaunated (○) continuous cultures

Table 3. Effect of cellulose loading rate on steady-state fermentation (means ±SD) in faunated and defaunated continuous cultures

| Determination | loading rate ($g.l^{-1}.d^{-1}$) | | | |
|---|---|---|---|---|
| | 16.3 | | 23.3 | |
| | F | DF | F | DF |
| VFA production ($mmol.l^{-1}.d^{-1}$) | 126±9 | 122±16 | 170±17 | 163±18 |
| Molar %, acetate | 70 | 71 | 72 | 74 |
| propionate | 22 | 22 | 19 | 19 |
| butyrate | 8 | 7 | 9 | 7 |
| pH | 6.53±0.24 | 6.55±0.28 | 6.60±0.22 | 6.59±0.19 |
| Methane production ($l.l^{-1}.d^{-1}$) | 1.7±0.2 | 1.8±0.3 | 2.8±0.4 | 2.8±0.4 |
| Ciliate number ($10^3.ml^{-1}$) | 65±3 | - | 72±7 | - |
| Bacterial number ($10^8.ml^{-1}$) | 120[c] | 178[c] | 229±33 | 366±69 |

a faunated
b defaunated
c n = 2

Table 4. Effect of substrate composition on steady-state ciliate species distribution (% of total) in continuous culture

| Group | Inoculum | Grass-grain | FPC[a] |
|---|---|---|---|
| *Diplodinium* | 13±4 | 45±7 | 86±5 |
| *Entodinium* | 68±3 | 31±6 | 8±4 |
| *Epidinium* | 3±1 | 24±2 | 6±3 |
| Holotrichs | 16±4 | 0 | 0 |

a filter paper cellulose

effect on ciliate numbers and NDF digestion in faunated continuous cultures (Gijzen et al, 1986). Fig 3 shows that NDF digestion in defaunated cultures was also positively correlated to dilution rate, but degradation efficiencies were lower as compared to faunated cultures at all dilution rates tested. However, the differences in NDF

degradation between faunated and defaunated cultures were smaller at lower dilution rates. At the same time, ciliate numbers in faunated cultures decreased markedly at lower diultion rates (Fig 3).

The effect of defaunation was also investigated with filter paper cellulose as a predominant substrate, fed at loading rates of 13 and 20 $g.l^{-1}.d^{-1}$, including 3.3 $g.l^{-1}.d^{-1}$ of ground alfalfa. In these experiments the addition of a small amount of alfalfa as an undefined source of nutrients appeared to be necessary (data not shown). Since the cellulose was completely degradable no solids were removed from the cultures, except for sample analyses. Dilution rate was kept at 2.0 $FV.d^{-1}$ for all experiments. This mode of operation resulted in a virtually complete digestion (98-100% of NDF) of the substrates at both loading rates, irrespective of the presence of ciliates.

Production of VFA and biogas were directly proportional to the loading rate applied and were similar in faunated and defaunated cultures (Table 3). Bacterial numbers were higher in defaunated cultures at both loading rates. Furthermore ciliate and bacterial numbers were higher at the high loading rate. Ciliate species composition of cellulose fed cultures differed markedly from grass fed cultures and from the inoculum (Table 4). These differences are probably a result of differences in substrate composition (Gijzen et al, 1987).

**Cellulase activity in faunated and defaunated cultures**

Besides overall degradation studies, also the distribution of cellulase activity was established in order to quantify the contribution of ciliates in fibre degradation. For this purpose faunated and defaunated cultures fed on filter paper cellulose were run parallel to each other. The cultures were maintained at a loading rate of 17 $g.l^{-1}.d^{-1}$ (including 2 g of alfalfa), while SRT and dilution rate were at 240 h and 2.0 $FV.d^{-1}$, respectively. A completely degradable substrate was used to eliminate the interference of residual substrate in the fractionation procedure.

Total cellulase activity, expressed as g $CMC.l^{-1}.d^{-1}$ (corresponding to about 0.1 mg $CMC.min^{-1}$. $mg^{-1}$ protein) of the defaunated culture was

Table 5. Cellulase activity [a] in various fractions of faunated and defaunated cultures fed on filter paper cellulose

| Fraction[b] | Faunated[c] (n = 5) | Parallel experiments[d] Faunated (n = 2) | Defaunated (n = 2) |
|---|---|---|---|
| TA | 215±39 | 231 | 169 |
| $F_1$ | 12±9 (7) | 3 (2) | 7 (5) |
| $F_2$ | 12±9 (7) | 12 (7) | 20 (13) |
| $F_3$ | 94±35 (58) | 127 (72) | 125 (82) |
| $F_4$ | 45±35 (28) | 35 (19) | 0 (0) |

a Cellulase activity is expressed as g CMC degraded.$l^{-1}$.$d^{-1}$ (within brackets: percentage of total activity)
b For explanation of fractions, see Fig 1
c Average (±SD) of 5 fractionations
d Average of two parallel fractionations

about 75% of the activity of the faunated one (Table 5). The difference can be ascribed to the absence of ciliates. The cellulase activity of the substrate-bound bacteria is about the same in both cultures, suggesting that cellulolytic bacteria do not account for the increased bacterial numbers usually observed in defaunated cultures. Under the conditions applied, the contribution of ciliates in cellulase activity is estimated to be about 19-28%.

## DISCUSSION

The results of in vivo studies are not consistant with respect to the role of ciliates in fibre degradation (Abou Akkada and El Shazly, 1964; Kurihara et al, 1978; Jouany and Senaud, 1979; Luther et al, 1966; Eadie and Gill, 1971; Bird and Leng, 1978). The experiments described in this study clearly demonstrate that degradation in faunated and defaunated cultures was strongly affected by loading and

dilution rates. Moreover Crawford et al (1980) demonstrated an effect of solid retention time on degradation and ciliate numbers in vitro. Since these parameters may vary widely in vivo, studies on faunted and defaunted ruminants should be regarded with caution.

In vitro conditions, on the other hand, are more standardized and reproducible, but the maintenance of high numbers of ciliates is required to establish the role of these organisms. An additional advantage of in vitro conditions is the possibility of using cycloheximide as a specific and efficient defaunating agens.

The results of in vitro digestibility studies with the grass-grain diet showed that the favourable effect of ciliates on overall digestion was most pronounced at high loading and dilution rates. NDF degradation efficiency at low dilution rates was only slightly higher in faunated cultures, probably as a result of the low ciliate numbers observed under these conditions. The enhancement of degradation efficiency in the presence of ciliates was most explicit for cellulose and lignin fibre fractions. Lignin degradation in the faunated cultures was more than twice as high as in the absence of ciliates. Although lignin degradation in the rumen is probably due to solubilization of low molecular fragments (Gaillard and Richards, 1975), these results suggest an active involvement of ciliates in apparent lignin digestion.

The lower bacterial numbers observed in faunated cultures are in agreement with previous reports (Kurihara et al, 1968; Eadie and Gill 1971) and probably result from the predatory action of ciliates on bacteria (Coleman, 1975). Because of this secondary effect of defaunation, it was not possible to establish the exact contribution of ciliates in overall fibre digestion.

Another approach in establishing the involvement of ciliates in plant cell wall degradation was made by determination of cellulase activity in various fractions of faunated and defaunated cultures. Although degradation was about complete both in the absence and presence of ciliates when filter paper cellulose was used as a predominant diet, total cellulase activity was higher in the faunated culture. In spite of the considerably higher number of bacteria in the defaunated culture bacterial cellulase activities in both cultures were about the same. This may be explained by the predatory action of

ciliates on bacteria, which was reported to favour the metabolic activity of bacteria (Kurihara et al, 1968; Demeyer and van Nevel, 1979). Under the conditions applied, ciliates accounted for about 19-28% of the total cellulase activity in the faunated culture.

At relatively low loading rates bacterial activity appeared to be sufficient to establish the same overall degradation as in the faunated situation. However, under conditions of high loading and dilution rates the presence of ciliates resulted in a significantly higher degradation. The results presented in this study indicate that ciliates contribute substantially in fibre degradation under all conditions applied.

## REFERENCES


Abou Akkada AR, El-Shazly K (1964) Effect of absence of ciliate protozoa from the rumen on microbial activity and growth of lambs. Appl Microbiol 12: 384-390

Akin DE, Amos HE (1979) Mode of attack on orchardgrass leaf blades by rumen protozoa. Appl Environ Microbiol 37: 332-338

Bird SH, Leng RA (1978) The effect of defaunation of the rumen on the growth of cattle on low-protein high-energy diets. Br J Nutr 40: 163-167

Coleman GS (1975) The interrelationship between rumen ciliate protozoa and bacteria. In: McDonald IW, Warner ACI (eds) Digestion and metabolism in the ruminant. Univ New Engl publ unit, Armidale, Australia, p149-164

Coleman GS (1986) The metabolism of rumen ciliate protozoa. FEMS Microbiol Rev 39: 321-344

Crawford RJ Jr, Hoover WH, Knowlton PH (1980) Effects of solids and liquids flows on fermentation in continuous culture. I, Dry Matter and fibre digestion, VFA production and protozoa numbers. J Anim Sci 51: 975-986

Demeyer DI (1981) Rumen microbes and digestion of plant cell walls. Agric Environ 6: 295-337

Demeyer DI, van Nevel CJ (1979) Effect of defaunation on the metabolism of rumen microorganisms. Br J Nutr 42: 515-524

Eadie JM, Gill JC (1971) The effect of the absence of rumen ciliate protozoa on growing lambs fed on a roughage-concentrate diet. Br J Nutr 26: 155-167

Gaillard BDE, Richards GN (1975) Presence of soluble lignin-carbohydrate complexes in the bovine rumen. Carbohydr Res 42: 135-145

Gijzen HJ, Lubberding HJ, Verhagen FJ, Zwart KB, Vogels GD (1987) Application of rumen microorganisms for an enhanced anaerobic degradation of solid organic waste materials. Agric Wastes, submitted

Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986) Continuous

cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol 25: 155-162
Goering HK, van Soest PJ (1970) Forage fiber analysis. USDA, ARS Agricultural Handbook no. 379
Hoover WH, Crooker BA, Sniffen CJ (1976) Effects of differential solid-liquid removal rates on protozoa numbers in continuous culture of rumen content. J Anim Sci 93: 528-535
Hungate RE (1966) The rumen and its microbes. Acad Press, New York
Jouany JP, Senaud J (1979) Role of rumen protozoa in the digestion of food cellulosic materials. Ann Rech Vet 10: 261-263
Jouany JP, Zainab B, Senaud J, Groliere CA, Grain J, Thivend P (1981) Role of the rumen ciliate protozoa Polyplastron multivesiculatum, Entodinium sp and Isotricha prostoma in the digestion of a mixed diet in sheep. Reprod Nutr Develop 21: 871-884
Kurihara Y, Eadie JM, Hobson PN, Mann SO (1968) Relationship between bacteria and ciliate protozoa in the sheep rumen. J Gen Microbiol 51: 267-287
Kurihara Y, Takechi T, Shibata F (1978) Relationship between bacteria and ciliate protozoa in the rumen of sheep fed on a purified diet. J Agric Sci 90: 373-381
Luther R, Trenkle A, Burroughs W (1966) Influence of rumen protozoa on volatile acid production and ration digestibility in lambs. J Anim Sci 25: 1116-1122
Orpin CG (1984) The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim Fd Sci Technol 10: 121-143
Rufener WH Jr, Nelson WO, Wolin MJ (1963) Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11: 196-201
Somogyi M (1952) Notes on sugar determination J Biol Chem 195: 19-23
Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21: 195-213
Williams AG, Strachan NH (1984) The distribution of polysaccharide degrading enzymes in the bovine rumen digesta ecosystem. Curr Microbiol 10: 215-220

CHAPTER 4

# HIGH-RATE TWO-PHASE PROCESS FOR THE ANAEROBIC DEGRADATION OF CELLULOSE, EMPLOYING RUMEN MICROORGANISMS FOR AN EFFICIENT ACIDOGENESIS

Gijzen HJ, Zwart KB, Verhagen FJ, Vogels GD,
(submitted for publication)

## SUMMARY

A novel two-stage anaerobic process for the microbial conversion of cellulose into biogas has been developed. In the first phase a mixed population of rumen bacteria and ciliates was used in the hydrolysis and fermentation of cellulose. The volatile fatty acids (VFA) produced in this acidogenic reactor were subsequently converted into biogas in a UASB-type methanogenic reactor.

A stepwise increase of the loading rate from 11.9 to 25.8 g volatile solids per liter reactorvolume per day (g $VS.l^{-1}.d^{-1}$) did not affect the degradation efficiency in the acidogenic reactor, whereas the methanogenic reactor appeared to be overloaded at the highest loading rate. Cellulose digestion was almost complete at all loading rates applied.

The two-stage anaerobic process was also tested with a closed fluid circuit. In this instance total methane production was 0.438 l $CH_4.g^{-1}$ VS added, which is equivalent to 98% of the theoretical value.

The application of rumen microorganisms in combination with a high-rate methane reactor is proposed as a means of efficient anaerobic degradation of cellulosic residues to methane. Because this novel two-phase system is based on processes and microorganisms of the ruminant, it will be referred to as 'rumen derived anaerobic digestion' (RUDAD) process.


## INTRODUCTION

Cellulosic biomass is produced as a solid waste product by agricultural and industrial activities, but on the other hand it may be considered as a potential resource for the production of biofuels like ethanol (Wiegel, 1982; Pieber and Toha, 1982) or biogas (Hobson, 1982; Hobson et al, 1984). Anaerobic digestion of cellulosic residues could provide an attractive means of waste reduction and waste stabilization with simultaneously recovery of methane as an energy source. However, in contrast to the situation with dissolved organic compounds present anaerobic treatments of solid organic waste materials lack high degradation efficiencies. Conversion of cellulose into biogas proceeds by

the action of various, metabolicly diverse populations of microorganisms, comprising hydrolytic, fermentative, hydrogen-producing acetogenic and methanogenic species (Zinder, 1984). Hydrolysis of cellulose is generally considered as the rate-limiting step in the overall degradation. Therefore an enhancement of the cellulolytic activity in bioreactors is of utmost importance for an economically feasible anaerobic degradation process.

A widespread natural cellulolytic system which operates with high efficiency is present in the rumen of ruminants (Wolin and Miller, 1983; Hungate, 1982). Several of the predominant bacteria and ciliates of this system are known tot exhibit high levels of cellulase activity, by which even crystalline cellulose can be hydrolysed (Russel, 1985; Coleman, 1983). In a previous paper we proposed the application of rumen microorganisms for an improved degradation of cellulosic residues and we have described a simple continuous cultivation system for rumen microorganisms, including ciliates (Gijzen et al, 1986). By the use of this system a high rate conversion of a grass-grain mixture into volatile fatty acids (VFA) and biogas could be obtained. The produced VFA should be removed efficiently in order to prevent acidification of the fermentation medium and to maintain a high degradation activity.

The present paper reports the high-rate two-phase conversion of filter paper cellulose into biogas at various loading rates. Hydrolysis and acidogenesis of the cellulose proceeded in the first reactor by the action of rumen microorganisms, whereas the produced VFA were subsequently converted into biogas in the second reactor. By placing the liquids of both reactors into serial interconnection a closed system was obtained which converts solid substrates into biogas.

## MATERIALS AND METHODS

### Acidogenic phase

Acidogenesis from cellulose was performed in a 3 liter volume continuous 'artificial rumen' fermenter as was described previously (Gijzen et al, 1986). Inoculation and operation conditions of the fer-

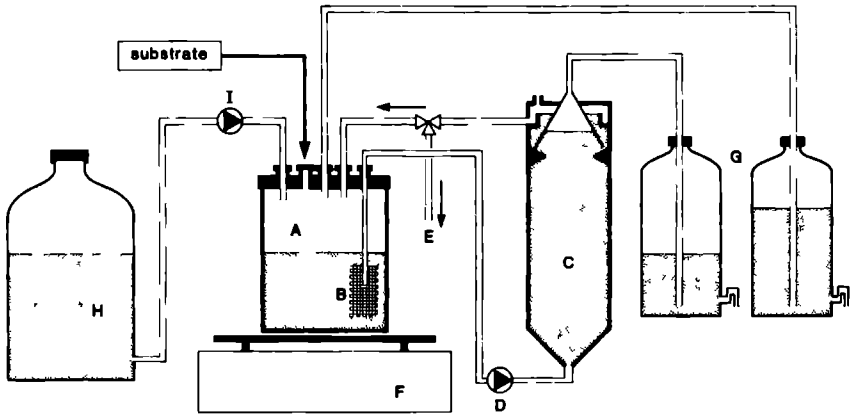



Figure 1. Schematic diagram of the two-stage anaerobic process used for the conversion of cellulose to methane. A) acidogenic reactor, B) 30 µm pore size filter, C) UASB-type methane reactor, D) filter effluent removal pump, E) methanogenic reactor effluent, F) rotary shaker, G) mariotte flask, H) fermentation medium reservoir, I) fermentation medium supply pump.

menter device were slightly modified. Buffer solution according to Rufener et al (1963) was modified by the addition of $NH_4HCO_3$ (2.2 $g.l^{-1}$) or $NH_4Cl$ (1.5 $g.l^{-1}$) as a nitrogen source and trace elements (0.2 $ml.l^{-1}$) according to Vishniac and Santer (1957). Experiments were started by inoculating fermenters with 250 ml strained rumen fluid obtained from a fistulated sheep. After inoculation the fermenters were filled with buffer solution to a working volume of 1.5 l. Buffer input and filtered effluent flow rates were adjusted to 2.0 fermenter volumes per day ($FV.d^{-1}$) for all experiments. No solids were withdrawn from the fermenters during the experimental period except for sample analysis. The substrate supplied to the fermenters consisted of filter paper cellulose (Whatman, grade 91), which was reduced to a particle size of 5 to 10 mm, supplemented with ground (1-2 mm) alfalfa hay (van Heeswijk, Veghel, the Netherlands) as an additional nutrient source. The substrate was added once daily, except the first day, when twice the normal daily amount was administered.

## Methanogenic phase

The acidogenic reactor was placed in serial connection with an up-flow anaerobic sludge blanket (UASB) methane reactor with a total volume of 2.5 l. The methanogenic reactor consisted of a glass cylinder (9 cm internal diameter, 28 cm height) with a water jacket, a conical shaped bottom (5 cm height) and a settler and gas collection compartiment. Biogas was collected by means of an inverted funnel construction in the gas collection compartiment. The volume of the gas collection compartiment was 0.5 l. The reactor was started by the use of about 1.25 l settled granular sludge (78 g VS.$l^{-1}$), which was obtained from a full scale potato processing waste treatment UASB-plant (AVEBE, de Krim, the Netherlands).

Liquid effluent from the acidogenic reactor, containing dissolved organic matter was continously fed to the methanogenic reactor at a rate of 3 $l.d^{-1}$. At this flow rate the hydraulic retention times (HRT) in the sludge layer and methanogenic reactor were about 10 and 20 h, respectively. The effluent of the methanogenic reactor was discharged in the tests on the effects of cellulose loading rates. To test the performance of a system with a closed fluid circuit, the effluent of the methanogenic reactor was continously fed to the acidogenic reactor. In this case the buffer supply was disconnected.

Both reactors were thermostatically controlled at 39°C by means of a temperature bath circulator. A schematic representation of the two-phase process is shown in Figure 1.

## Experimental conditions

The effect of loading rate on the two-phase digestion process was studied in four short-term experiments of 35 days each. Acidogenic space loading rates were 10, 15, 20 and 25 g $l^{-1}.d^{-1}$ of filter paper cellulose, supplemented with 3.33 g.$l^{-1}.d^{-1}$ of alfalfa hay. The chemical composition of the substrates is shown in Table 1. $NH_4HCO_3$ was used as a source of nitrogen in all experiments, except at the lowest loading rate, where $NH_4Cl$ was used. The effect of recirculation of the second phase effluent was investigated during a fermentation period of 68 days at a loading rate of 15 g.$l^{-1}.d^{-1}$ of filter paper cellulose

Table 1. Composition of the substrates[a]

| Determination | | Alfalfa hay | Filter paper cellulose |
|---|---|---|---|
| Dry weight | (%) | 92.3 ± 0.6 | 93.9 ± 0.1 |
| VS | (% of dw[b]) | 87.4 ± 0.9 | 99.8 ± 0.1 |
| Ash | (% of dw) | 12.6 ± 0.9 | 0.2 ± 0.1 |
| NDF | (% of dw) | 43.8 ± 0.2 | n.d.[c] |
| ADF | (% of dw) | 31.5 ± 0.7 | n.d. |
| Hemicellulose | (% of dw) | 12.3 ± 0.6 | n.d. |
| Cellulose | (% of dw) | 21.8 ± 0.2 | 98.5 |
| Lignin | (% of dw) | 9.7 ± 0.9 | n.d. |
| Cell-solubles | (% of dw) | 43.6 ± 0.6 | 1.3 |
| COD[d] | (g $O_2.g^{-1}$ dw) | 1.08 ± 0.04 | 1.22 ± 0.02 |

a Means ± standard deviation (SD)

b Dry weight

c Not determined

d Chemical oxygen demand

and 3.33 $g.l^{-1}.d^{-1}$ of alfalfa hay. During this expriment $NH_4Cl$ was used as a nitrogen source in the fermentation medium. Complete fluid recirculation within the two-phase process was started 19 days after inoculation.

**Sampling and analyses**

Samples of 10 ml were withdrawn from the acidogenic reactor content and from the methanogenic reactor effluent three times a week for determination of pH, protozoal numbers (acidogenic phase only) and VFA concentration. During the experiments performed at different loading rates, sampling was started one week after inoculation, after which a steady-state situation was reached (Gijzen et al, 1986). The samples were taken 4 hours after substrate addition, because preliminary experiments (data not given) had shown that the values obtained at this time approximate mean values of a 24 h fermentation period. Protozoal

numbers and species distribution and concentrations of individual VFA were determined as described previously (Gijzen et al, 1986).

Biogas production of both acidogenic and methanogenic reactors were measured daily by means of a mariotte flask containing acidified tap water (about 0.02% HCl). Methane content of the biogas was determined once every week in duplo according to Hutten et al (1981).

Samples for determination of protein (1 ml) and coenzyme F420 (4 ml) were taken from the acidogenic reactor once a week. Extraction and HPLC analysis of coenzyme F420 were performed according to the method of Gorris and van der Drift (1986). Proteins in 1 ml homogeneous samples were extracted by the addition of 0.5 ml 1.5 N NaOH and heating for 30 min in boiling water. After addition of 0.5 ml 1.5 N HCl and 0.2 ml 0.15% sodiumdeoxycholate the samples were thoroughly mixed and allowed to stand for 30 min at room temperature. The samples were centrifuged at 7000 g for 15 min and the protein present in the supernatants was precipitated with trichloroacetic acid. The precipitates obtained after centrifugation (7000 g, 10 min) were suspended in 1 ml 0.5 N NaOH and protein was estimated by the biuret method according to Herbert et al (1971). Pellets obtained after the first centrifugation step were extracted for a second time according to the whole procedure and protein content obtained was added to that obtained after the first extraction.

Neutral detergent fibre (NDF), acid detergent fibre (ADF), cellulose, hemicellulose and lignin content of the substrates were determined according to the method of Goering and van Soest (1970). Cell solubles were defined as VS minus NDF (Chandler et al, 1980).

## RESULTS

### Cellulose degradation at different loading rates

Preliminary experiments indicated that the buffer capacity of the fermentation medium with $NH_4Cl$ as a nitrogen source was sufficient at loading rates up to 16.6 g $VS.l^{-1}.d^{-1}$. However, at higher loading rates a temporary acidification (pH < 5.7) and subsequent accumulation of cellulose in the acidogenic reactor occurred. The acidifica-

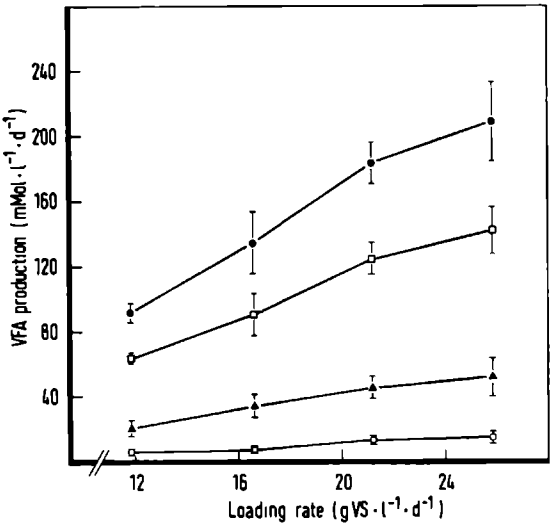



Figure 2. Effect of cellulose loading-rate on the production of acetate ( □ ), propionate ( ▲ ), butyrate ( o ) and total VFA ( ● ) in the acidogenic reactor. All values are expressed as means (± SD) during steady-state performance.

Table 2. Effect of loading-rate on pH in acidogenic and methanogenic reactor effluents

| Loading-rate g VS.$l^{-1}$.$d^{-1}$ | pH[a] acetogenic reactor | methanogenic reactor |
|---|---|---|
| 11.9 | 6.6 ± 0.2 | 7.5 ± 0.1 |
| 16.6 | 6.6 ± 0.2 | 7.8 ± 0.1 |
| 21.2 | 6.3 ± 0.2 | 7.8 ± 0.2 |
| 25.8 | 6.1 ± 0.2 | 7.4 ± 0.4 |

[a] Mean valves ±SD

tion resulted in irreversible effects on the fermentation process. Therefore $NH_4Cl$ was replaced by $NH_4HCO_3$ at all loading rates except

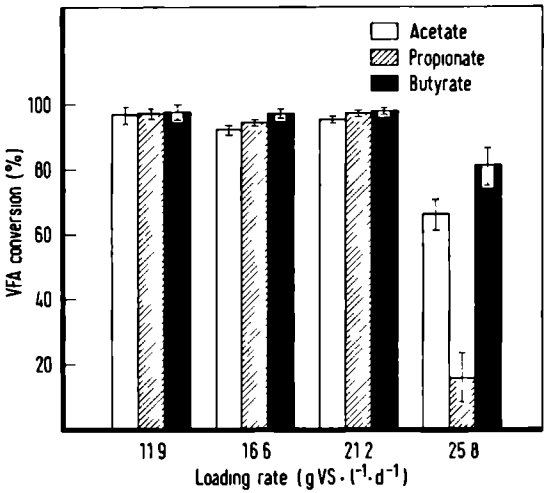



Figure 3. Effect of cellulose loading-rate on VFA conversion in the methanogenic reactor. All values are expressed as means (± SD) during steady-state performance.

11.9 g $VS.l^{-1}.d^{-1}$, resulting in a higher buffer capacity of the fermentation medium.

The effect of different loading rates in the acidogenic reactor on various fermentation parameters was established in 4 experiments. Each experiment lasted 35 days, including 7 days stabilization time. The average pH of the acidogenic reactor remained between 6.1 and 6.6 at all loading rates (Table 2). During the entire 35 days of continuous operation, less than 0.5% of the total solid substrate input was removed from the acidogenic reactors by means of sampling for analyses. Since no significant accumulation of substrates could be observed at any of the loading rates applied, digestion in the acidogenic reactors was considered to be virtually complete. Figure 2 shows the mean steady-state data on the production of VFA (concentration x dilution rate) in the acidogenic reactor as a function of cellulose feeding rate. Total VFA production increased almost proportionally with increasing loading rate, but the production lags slightly behind at the highest loading rate. At all loading rates applied the molar percentages of acetate, propionate and butyrate were about 68%, 25% and 7%, respectively. pH values were affected by the increase of VFA concen-

tration in the acidogenic reactors (Table 2). Average degradation efficiencies of total VFA in the methanogenic reactor varied between 93% and 97% at loading rates up to 21.2 g $VS.l^{-1}.d^{-1}$, but dropped to 53% at the highest loading rate applied (Figure 3). At all loading rates, butyric acid exhibited the highest conversion. The low conversion efficiency of VFA at the highest loading rate was attributable to propionic acid, which passed the methanogenic reactor almost unaffected.

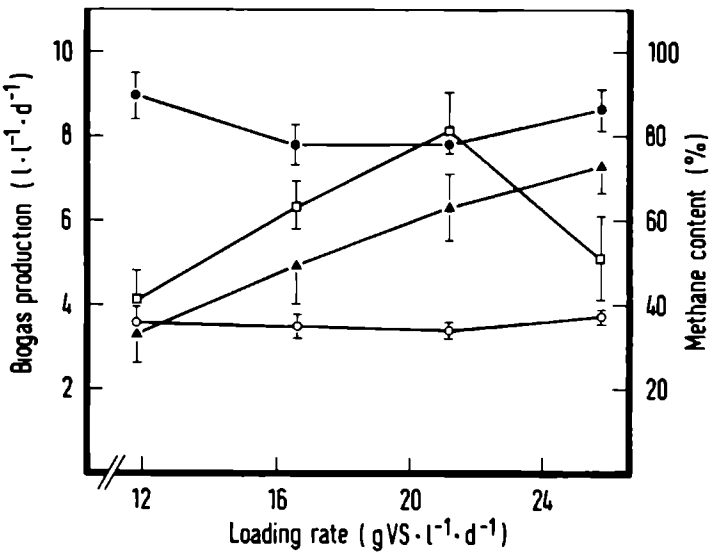



Figure 4. Effect of cellulose loading-rate on biogas production and methane content of the biogas from the acidogenic and methanogenic reactors. ( ▲ ) biogas from acidogenic reactor, ( □ ) biogas from methanogenic reactor, ( o ) $CH_4$ content of biogas from acidogenic reactor, ( ● ) $CH_4$ content of biogas from methanogenic reactor. All values are expressed as means (± SD) during steady-state performance.

Biogas and methane productions in the acidogenic reactor showed a close correlation to the loading rate applied (Figure 4). A proportional increase of methane production in the methanogenic reactor was observed at loading rates up to 21.2 g $VS.l^{-1}.d^{-1}$. The specific gas

Table 3. Effects of cellulose loading-rate on microbial populations in the acidogenic reactor. All values are expressed as means (±SD) during steady-state performance.

| Loading-rate | ciliates | Species distribution (% of total) | | | Protein | Coenzyme F420 |
|---|---|---|---|---|---|---|
| g VS $l^{-1}.d^{-1}$ | $10^3.ml^{-1}$ | *Diplodinium* | *Epidinium* | *Entodinium* | $g.l.^{-1}$ | $\mu mol.l^{-1}$ |
| 11.9 | 72 ± 13 | 83 ± 13 | 12 ± 4 | 5 ± 3 | 3.5 ± 0.4 | 0.43 ± 0.12 |
| 16.6 | 84 ± 12 | 81 ± 7 | 6 ± 1 | 13 ± 6 | 4.1 ± 0.3 | 0.45 ± 0.02 |
| 21.2 | 93 ± 15 | 90 ± 3 | 5 ± 2 | 5 ± 3 | 5.2 ± 0.4 | 0.41 ± 0.07 |
| 25.8 | 71 ± 12 | 95 ± 13 | 2 ± 1 | 3 ± 1 | 4.0 ± 0.9 | 0.43 ± 0.10 |
| inoculum | 268 ± 44 | 20 ± 5 | 3 ± 2 | 64 ± 4 | n.d. | n.d. |

production ($l.g^{-1}$ VS) decreased significantly at the highest loading rate due to the incomplete VFA conversion. The methane content of the biogas from acidogenic and methanogenic reactors varied between 35-40% and 78-90%, respectively.

The total number of rumen ciliates and their species distribution in the acidogenic reactor were not affected by loading rate (Table 3). The ciliates present in the inoculum belonged mainly to the *Entodinium* group, but within 6 to 8 days after inoculation ciliates of the *Diplodinium* group were predominant in the cellulose-fed reactors. Holotrich ciliates were present in high numbers in the inoculum (12%), but disappeared within a few days after inoculation. The total ciliate number dropped by a factor 3 as compared to the inoculum, but in comparison of the ciliate biomass one has to consider that ciliates belonging to the *Diplodinium* group represent a much higher biomass content than ciliates of the *Entodinium* group.

Protein content in the acidogenic reactors remained rather constant at all loading rates, suggesting that microbial biomass was similar in all experiments. Concentrations of coenzyme F420, indicative for the number of methanogens, also remained rather constant at different loading rates (Table 3).

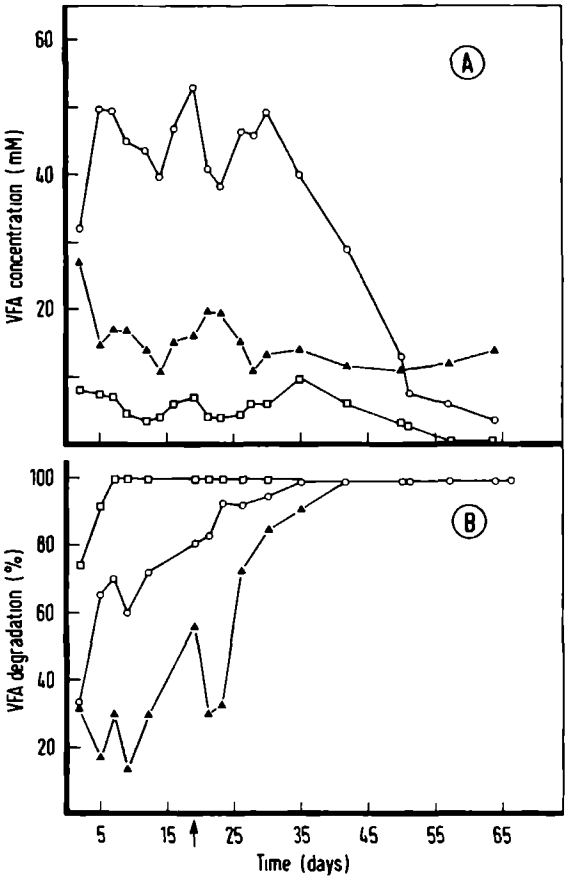



Figure 5. Effect of prolonged fluid recirculation on the concentrations of acetate ( o ), propionate ( ▲ ) and butyrate ( □ ) in the acidogenic reactor (A) and their conversion efficiency in the methanogenic reactor (B). Arrow indicates the start of fluid recirculation.

**Effect of fluid recirculation**

The effect of fluid recirculation was established during a 68 days fermentation period at a loading rate of 16.6 g $VS.l^{-1}.d^{-1}$ without removal of solids, except for sampling. After day 19 of operation no fluid was removed from or added to the two-phase system anymore. The

substrate conversion during the entire experimental period was estimated to be almost complete. Only a slight accumulation of alfalfa hay residues could be observed after several weeks of operation.

The fermentation process in the acidogenic reactor was rather stable during the first 30 days of operation, as assessed by VFA concentration (Figure 5A) and biogas production (Figure 6A). VFA and biogas production during this period were comparable to those observed during the loading rate experiment at 16.6 g $VS.l^{-1}.d^{-1}$ substrate addition. Average VFA production during this period amounted to 135 ± 11 (SD) $mmol.l^{-1}.d^{-1}$ with molar ratios of 70%, 21% and 9% for acetate, propionate and butyrate, respectively.

From day 30 of operation on several changes were observed in the fermentation pattern. The concentration of acetate decreased gradually, until a value of approximately 5 mM was reached at day 50 of operation (Figure 5A). Butyric acid disappeared in the acidogenic reactor, but the concentration of propionic acid remained unchanged during the entire experimental period. As a consequence of the lower VFA concentration the pH of the acidogenic reactor content gradually increased from 5.8 to about 7.0 (Figure 7). The decrease of total VFA concentration was accompanied by a simultaneous increase of both biogas production and methane content of the biogas (Figure 6A). Obviously acetate and butyrate were converted into biogas already in the acidogenic reactor by the action of butyrate oxydizing acetogenic bacteria together with acetoclastic and hydrogenotrophic methanogenic bacteria. This process was probably induced by a transfer of non-flocculant bacteria from the methanogenic reactor and subsequent growth in the acidogenic reactor. The increase in methane concentration of the biogas observed after recirculation of the fermentation medium was probably a result of a relative decrease of $CO_2$ evolution at the increased pH of the acidogenic reactor content.

The drastic changes in the fermentation pattern indicated that recirculation of the fermentation medium had markedly affected microbial populations in the acidogenic reactor. Epifluorescence microscopical observations demonstrated that during the first 30 days after inoculation only small rod-shaped methanogens were present, presumably *Methanobrevibacter ruminantium*, a hydrogenotrophic species generally present in the rumen. However, after proglonged recirculation *Methano-*

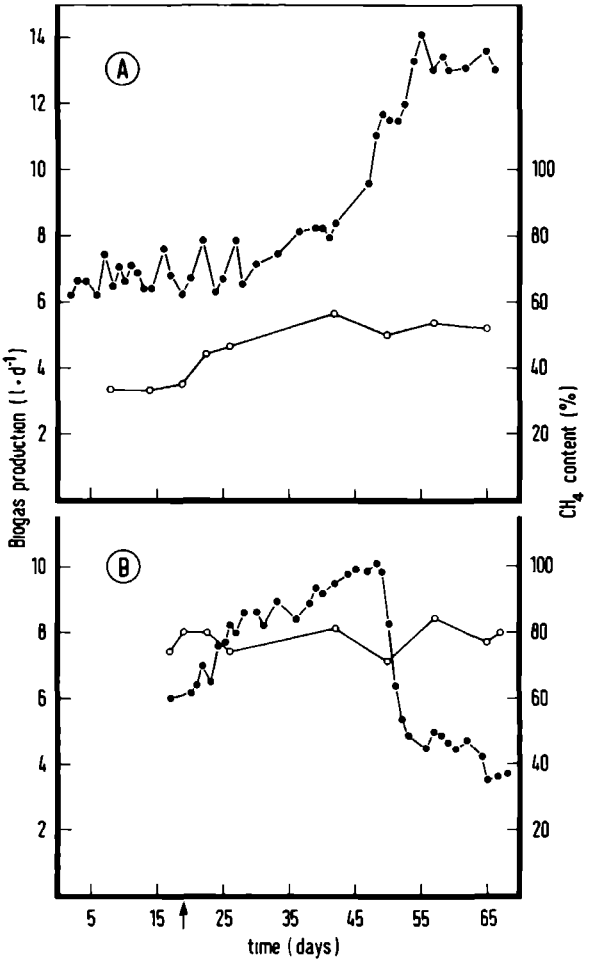



Figure 6. Effect of prolonged fluid recirculation on biogas production ( ● ) and methane content of biogas ( o ) in the acidogenic (A) and methanogenic reactors (B). Arrow indicates start of recirculation.

*sarcina* species had accumulated. Apart from the composition of microbial species also the total number of microorganisms was affected by recirculation, as indicated by the increase of protein cocnentration in the acidogenic reactor (Figure 8). Protozoal numbers increased drasticly immediately after recirculation was started, but then the

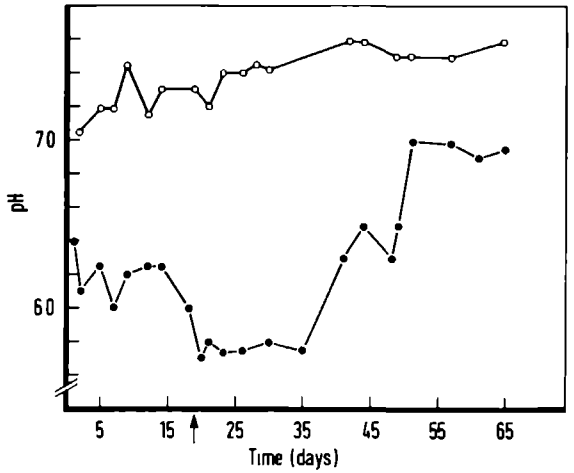



Figure 7. Effect of prolonged recirculation of the fermentation medium on pH of acidogenic ( ● ) and methanogenic ( o ) reactor effluents. Arrow indicates the start of fluid recirculation.

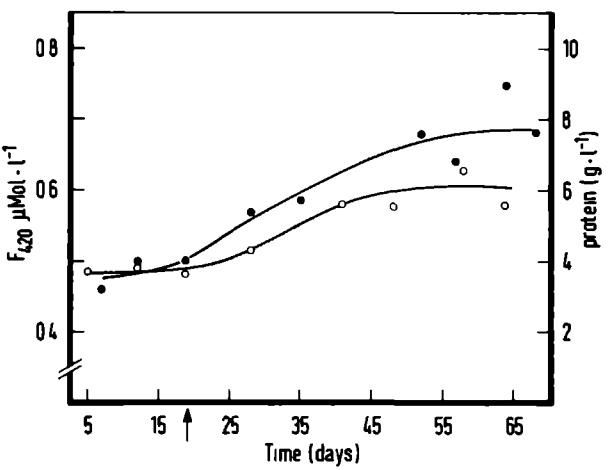



Figure 8. Effect of prolonged recirculation of the fermentation medium on protein ( o ) and coenzyme F420 ( ● ) concentrations of the acidogenic reactor. Arrow indicates the start of fluid recirculation.

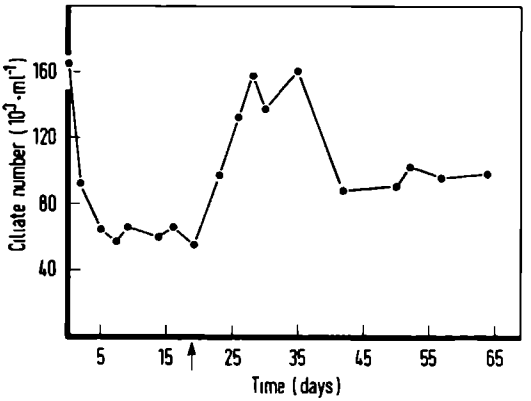



Figure 9. Effect of prolonged recirculation of the fermentation medium on protozoal numbers in the acidogenic reactor. Arrow indicates the start of recirculation.

number decreased again to a steady-state level of approximately $10^5$ cells.ml$^{-1}$ (Figure 9). The relative number of different protozoal species were not affected by fluid recirculation and were comparable to those found during the loading-rate experiments (Figure 10). Figure 8 shows the course of coenzyme F420 concentration in the acidogenic reactor. The observed increase of F420 indicates an increase in the number or activity of methanogens as a result of recirculation (Gorris and van der Drift, 1986).

The performance of the methanogenic reactor used in this experiment was poor during the first days of operation, probably as a result of storage of the granular sludge at 4°C for a period of several weeks before the start of the experiment. VFA conversion gradually improved until an almost complete conversion (94%) was reached after 30 days of operation (Figure 5B). The improved conversion efficiency resulted in an increased effluent pH (Figure 7) and biogas production (Figure 6B). However, after prolonged recirculation biogas production decreased drasticly as a consequence of lack of substrate supply due to the almost complete degradation of acetate and butyrate in the acidogenic reactor. The methane content of the biogas remained unchanged at 78 ± 4% (SD) during the entire experimental period.

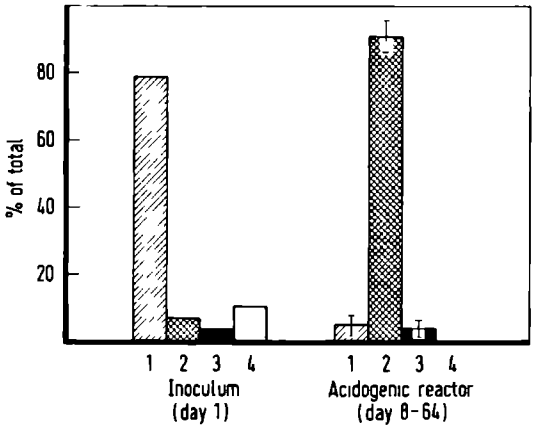



Figure 10. Effect of prolonged recirculation on the relative number of protozoa belonging to the groups of *Entodinium* (1), *Diplodinium* (2), *Epidinium* (3) and Holotrichs (4) in the acidogenic reactor (means ± SD).

## DISCUSSION

The results of this study show that the combination of an acidogenic reactor exhibiting high cellulolytic activities due to the presence of rumen microorganisms, and a high-rate UASB-type methane reactor gives rise to an efficient anaerobic degradation of cellulose into biogas. At all loading-rates of the acidogenic reactor from 11.9 up to 25.8 g $VS.l^{-1}.d^{-1}$ the degradation of the substrate was almost complete, since only a slight accumulation of alfalfa residues were observed after prolonged periods of operation. The specific productions of methane and VFA in the acidogenic reactor were almost constant with mean values of 0.1 $l.g^{-1}VS$ and 8.2 $mmoles.g^{-1}VS$, respectively. These results indicate that the fermentation process in the acidogenic reactor is not affected by loading-rate in the range tested.

Up to a loading-rate of 21.2 g $VS.l^{-1}.d^{-1}$ the VFA produced in the acidogenic reactor were almost completely converted into biogas by the action of the UASB methanogenic reactor on the effluent of the acido-

genic reactor. However, the specific gas production of the methanogenic reactor decreased significantly at the highest loading-rate (25.8 g $VS.l^{-1}.d^{-1}$) and the absolute amount of gas produced and VFA converted were even lower as compared to the results obtained at a loading rate of 21.2 g $VS.l^{-1}.d^{-1}$. This suggests that overloading of the methanogenic reactor results in an inhibition of VFA conversion into biogas. Propionate conversion appeared to be most strongly inhibited, probably because propionate oxidation is thermodynamically rather unfavorably in anaerobic digestion (Zehnder and Koch, 1983). Moreover, the elevated acetate concentration in the methanogenic reactor may involve product inhibition in the degradation of propionate and butyrate (de Zeeuw, 1984).

From the equation according to Buswell and Mueller (1952),

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right) H_2O \longrightarrow \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right) CO_2 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right) CH_4$$

and by assuming the organic part of the substrate added to the acidogenic reactor to be $(C_6H_{10}O_5)n$, the combined methane productions of acidogenic and methanogenic reactors were calculated to be 93, 91, 89 and 63% of the theoretical maximum values at loading-rates of 11.9, 16.6, 21.2 and 25.8 g $VS.l^{-1}.d^{-1}$, respectively. The balance may be accounted for by cell growth and incomplete conversion of VFA at the highest loading-rate.

A conversion rate close to 25.8 g $VS.l^{-1}.d^{-1}$ as obtained with this novel two-phase process is significantly higher than conversion rates reported for other anaerobic systems (Khan et al, 1983; Laube and Martin, 1981; Petitdemange et al, 1984) dealing with cellulose as a substrate. In fact this loading-rate is comparable with those reported for advanced high-rate methanogenic reactors treating easily degradable soluble wastes (van den Berg, 1984). The high rate of cellulose hydrolysis could be maintained and acidification could be prevented by removal of the fermentation fluids, which were applied to a separate high-rate methanogenic reactor in order to obtain an overall degradation into biogas.

The results demonstrate that the process operates quite satisfac-

tory with a closed fluid circuit in which the effluent of the methanogenic reactor flows back into the acidogenic reactor. In this set-up the methanogenic reactor functions as a pH-buffering system as well. After prolonged recirculation of the fermentation medium, part of the methanogenic activity had been taken over by the acidogenic reactor probably due to a distribution of non-flocculant methanogenic bacteria over both reactors. In this situation the methanogenic reactor is still to a minor extent effective as a pH regulating system (Figure 7) and its main function consists of propionate degradation (Figure 5). Propionate degradation was not observed in the acidogenic reactor. Probably the experimental period was still too short to build up a propionate oxidizing flora in the acidogenic reactor, because of the long generation time of the organisms involved (Zehnder and Koch, 1983). Alternatively the spatial separation of propionate degradation may reflect a prerequisite of complete VFA degradation in this closed fluid system due to the presence of acetate and possible hydrogen at concentrations still inhibitory in the acidogenic reactor.

As a result of the substantial VFA conversion in the acidogenic reactor pH increased and acidification is less likely to occur. As a consequence the fluid turnover rate may even be reduced further than applied in this study. Such a reduction would be attractive in a large scale application of the process. In the process with a closed fluid circuit a situation was met in which cellulosic substrates were the only input and biogas was the only output of the two-phase system. Total methane production of both reactors yielded up to 98% of the theoretical maximum value. This methane yield was significantly higher than that observed during the loading-rate experiments. The higher methane yield was obtained under conditions where no microbial biomass and fermentation products (VFA) were removed from the system. During the period between day 19 to day 68 after inoculation microbial and methanogenic biomass in the acidogenic reactor had increased somewhat as indicated by the amounts of protein and coenzyme F420 present. However, the total amount (813 $g.l^{-1}$) of cellulose converted during that period was out of all proportion to the observed increase of biomass. Cellulose conversion to biogas had proceeded apparently as a largely growth-independent process, possibly due either to uncoupling at a molecular and physiological level or to effects involving cells an po-

pulations, which eat each other. Further studies are needed to explain this striking result.

The two-phase process described here will be referred to as the 'rumen derived anaerobic digestion' (RUDAD-) process. Application of this two-phase process could have major implications on the economical feasibility of industrial scale anaerobic digestion of cellulosic waste materials.

## REFERENCES


Buswell AM, Mueller HF (1952) Mechanisms of methane fermentation. Indust Eng Chem 44: 550-552

Chandler JA, Jewell WJ, Gossett JM, van Soest PJ, Robertson JB (1980) Predicting methane fermentation biodegradability. Biotechnol Bioeng Symp 10: 93-107

Coleman GS (1983) The cellulolytic acitivity of thirteen species of rumen entodiniomorphid protozoa. J Protozool 30: 36a

de Zeeuw W (1984) Acclimatization of anaerobic sludge for UASB-reactor start-up. PhD Thesis, Agricultural University of Wageningen, the Netherlands

Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986) Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol, in press

Goering HK, van Soest PJ (1970) Forage fiber analysis. USDA, ARS agricultural handbook no. 379

Gorris L, van der Drift C (1986) Methanogenic cofactors in pure cultures of methanogens in relation to substrate utilization. In: Proc Int seminar on biology of anaerobic bacteria, Lille, France 17-18 June, in Press

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. In: Norris JR, Ribbons DW (eds) Methods in Microbiology, 5B, Academic Press, London, New York, p209-344

Hobson PN (1982) Biogas production from agricultural wastes. Experientia 38: 206-209

Hobson PN, Reid GW, Sharma VK (1984) Anaerobic conversion of agricultural wastes to chemicals or gases. In: Ferrero GL, Ferranti MD, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste. Elsevier applied science publishers, London, New York, p.369-381.

Hungate RE (1982). Methane formation and cellulose digestion, biochemicalecology and microbiology of the rumen ecosystem. Experientia 38: 189-192

Hutten TJ, de Jong MH, Peeters BPH, van der Drift C, Vogels GD (1981). Coenzyme M (2-mercapto enthane sulfonic acid) - derivates and their effects on methane production from carbon dioxide and methanol by cell-free extracts of methanosarcina barkeri. J Bacteriol 145: 27-34

Khan AW, Miller SS, Murray WD (1983) Development of a two-phase combination fermentor for the conversion of cellulose to methane.

Biotechnol Bioeng 25: 1571-1579
Laube VM, Martin SM (1981). Conversion of cellulose to methane and carbon dioxide by triculture of acetivibrio cellulolyticus, Desulfovibrio sp and methanosarcina barkeri. Appl Environ Microbiol 42: 413-420
Noike T, Endo G, Chang JE, Yaguchi JI, Matsumoto JI (1985) Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion. Biotechnol Bioeng 27: 1482-1489
Petitdemange A, Fond D, Ravel G, Petitdemange H, Gay R (1984) Fermentation of cellulose by a coculture of Clostridium cellulolyticum and Clostridium acetobutylicum. In: Ferrero GL, Ferranti MD, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste. Elsevier applied science publishers, London, New York, p223-234
Pieber M, Toha JC (1982) A general view of ethanol and methane production from cellulosic residues. J Ferment Technol 60: 247-252
Rufener WH Jr, Nelson WD, Wolin MJ (1963). Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11: 196-201
Russell JB (1985). Fermentation of cellodextrins by cellulolytic and non-cellulolytic rumen bacteria. Appl Environ Microbiol 49: 572-576
Van den Berg L (1984). Developments in methanogenesis from industrial waste water. Can J Microbiol 30: 975-990
Vishniac W and Santer M (1957). The Thiobacilli Bacteriol Rev 21: 195-213
Wiegel J (1982) Ethanol from cellulose. Experientia 38: 151-156
Wolin MJ, Miller TM (1983). Interactions of Microbial populations in cellulose fermentation. Federation proceedings 42: 109-113
Zehnder AJB, Koch ME (1983). Thermodynamic and kinetic interactions of the final steps in anaerobic digestion. In: Proc Eur Symp anaerobic waste water treatment, Noordwijkerhout, the Netherlands, p86-96
Zinder SH (1984). Microbiology of anaerobic conversion of organic wastes to methane: recent developments. ASM News 50: 294-298.

# APPLICATION OF RUMEN MICROORGANISMS FOR AN ENHANCED ANAEROBIC DEGRADATION OF SOLID ORGANIC WASTE MATERIALS

Gijzen HJ, Lubberding HJ, Verhagen FJ, Zwart KB, Vogels GD,
(submitted for publication)

## SUMMARY

Organic waste materials of various origin and composition have been screened on anaerobic digestion by a mixed population of rumen microorganisms. High loading rates could be applied, varying between 17.1 (coffee pulp) and 32.4 g (papermill sludge) of volatile solids per litre per day ($VS.l^{-1}.d^{-1}$) at retention times of only 60 h. Degradation efficiencies obtained for most waste material were in the range of 60-70%, irrespective of their chemical composition. During the degradation of lignocellulosic waste materials an apparent lignin degradation up to 48% was observed. Average productions of volatile fatty acids and biogas per g VS digested varied between 6.6 to 8.2 mmoles and 0.18 to 0.29 litre (approximately 40% $CH_4$), respectively for most waste materials. Molar proportions of butyrate were higher with substrates rich in cell solubles, as compared to cellulosic and lignocellulosic substrates which yielded more propionate. Rumen ciliates belonging to the *Entodinium* group were predominant during the fermentation of substrates rich in cell solubles, whereas ciliates of the *Diplodinium* group were predominant with cellulosic and lignocellulosic substrates.

## INTRODUCTION

An excess of organic waste containing large amounts of cellulose is produced worldwide mainly by agricultural, municipal and food-processing activities. The increasing production of these waste materials has raised the need for economically viable disposal methods. Especially for waste materials with a relatively high moisture content, an anaerobic degradation process might be preferable to more traditional disposal methods like dumping or incineration. Major benefits of anaerobic digestion are: the reduction of waste volume, smell and pathogenic organisms, the production of biogas, and the potential use of the remaining residue as a soil conditioner or fertilizer. An important drawback of current anaerobic digesters however is formed by the slow and incomplete degradation of solid organic wastes.

Depending on the nature of the waste material, diferent reasons can

be given for the slow conversion rates. If the predominant constitutents of the organic waste consist of easy degradable materials (e.g. starch, soluble sugars), acetogenesis and methanogenesis have been identified as the rate-limiting steps, because of the relatively long generation times of the organisms involved (Ghosh and Pohland, 1974; Kaspar and Wuhrmann, 1978). However if cellulose is a major constituent of the waste material, its hydrolysis has shown to be rate-limiting in overall anaerobic digestion (Noike et al, 1985). The rate of hydrolysis is primarily dependent on the crystallinity of the cellulose, the degree of association with lignin (Ladisch et al, 1983; Cowling and Kirk, 1976) and the cellulase activity of the microbial process applied.

Recently we demonstrated that the application of rumen microorganisms, in combination with a high-rate UASB-type methane reactor, resulted in an enhanced conversion of pure cellulose into biogas (Gijzen et al, 1987). The improved conversion rate resulted from the high cellulase activity of the unique rumen microbial population applied in this system. This microbial system has a potential application to the anaerobic degradation of lignocellulosic waste materials, but has not yet been tested for this purpose. This study demonstrates the potentials of the newly developed process and presents an overview of the degradation of organic waste materials of various origin and composition by the action of rumen microorganisms in an acidogenic reactor.

## MATERIALS AND METHODS

### Digester feed

Various organic waste materials of agricultural, industrial and municipal origin were used as a substrate for anaerobic digestion. The waste materials tested for degradation include barley straw, bagasse, verge grass, coffee pulp (peel and mesocarp of the coffee bean), partially dehydrated papermill sludge, papermill pulp reject (pulping residue), vegetable auction waste (VAW; vegetables not fetching the minimum price), horticultural waste, spent mushroom compost (SMC), a cellulosic fraction of municipal solid waste (MSW), usually referred

to as refuse derived fuel (RDF) and a fine organic fraction of MSW (MSW-OF). The MSW fractions were obtained from a mechanical separation system for municipal refuse. On the basis of the known amounts of VAW in the Netherlands, we composed a mixture of 44% tomatoes, 17.5% cucumbers, 17.5% lettuce, 12% endive and 9% pears (on wet weight basis). The horticultural waste was composed of 33% tulip stalks and bulbs, 33% chicory tubers, 17% mushroom remnants and 17% turnips (on wet weight basis).

All waste materials, except VAW and papermill sludge, were dried (16 h, 70°C) and ground immediately at arrival. Before grinding the MSW fractions most of the plastics, stones and glass were separated by hand. Plastics present in the papermill pulp (approximately 50% of dry weight) were carefully separated by hand. Papermill sludge and VAW were stored at -20°C, whereas the dried materials were stored at room temperature.

In addition to the waste materials, alfalfa hay *(Medicago sativa)*, purchased from van Heeswijk (Veghel, the Netherlands) and filter paper cellulose (Whatman, grade 91) were used as model substrates for anaerobic degradation. The chemical composition of waste materials and model substrates used in this study is shown in Fig 1 and Table 1.

**Fermenter design and operation**

Experiments were performed in 3 l fermenters (1.5 l working volume) operated with differential removal rates of solids and liquids, as was described previously (Gijzen et al, 1986). The fermenters were inoculated with 250 ml fresh strained rumen fluid, which was obtained from a sheep providied with a rumen fistula. After inoculation the reactors were filled with pre-warmed (39°C) fermentation medium according to Rufener et al (1963). During some experiments, as indicated in Table 2, the fermentation medium was modified by the addition of $NH_4Cl$ (1.5 $g.l^{-1}$) as a source of nitrogen and trace elements (0.2 $ml.l^{-1}$) according to Vishniac and Santer (1957). Digester feed was added once a day except at the day of inoculation, when twice the normal loading rate was administered. during some experiments a small amount of alfalfa was added as an additional source of nutritients.

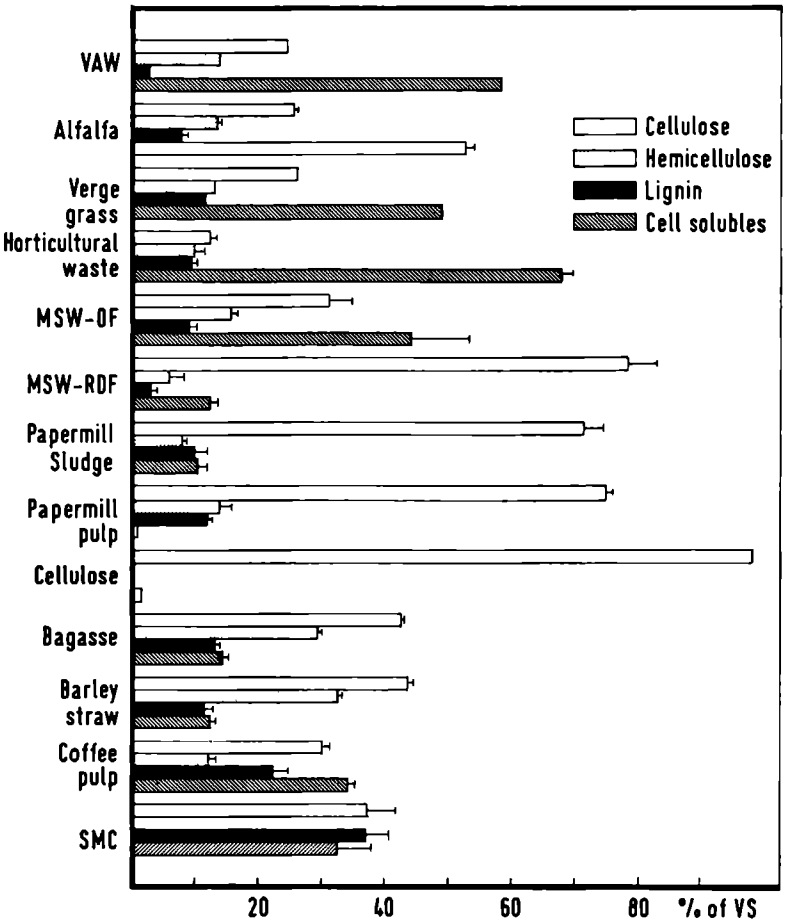



Fig 1. Cell wall carbohyrate composition and cell solubles content of different types of digester feed (means ±SD)

**Experimental conditions**

All fermentations were carried out over periods of at least two weeks, including a 7 days adaptation period. Steady state conditions were reached within a period of 5-7 days, as assessed by fermentation characteristics. The fermenters were kept at a temperature of 39°C.

The extent of decomposition of the waste materials was measured

TABLE 1. Chemical composition of substrates for anaerobic digestion

| Substrate | TS (%) | VS (% of TS) | NDF (% of TS) | COD ($gO_2.g^{-1}$TS) | N (% of TS) |
|---|---|---|---|---|---|
| Group A | | | | | |
| VAW | 5.7±0.1 | 90.1±0.5 | 34.2 | 1.15±0.08 | 1.93±0.07 |
| Alfalfa | 93.9±0.9 | 92.4±1.0 | 47.3 | 1.08±0.04 | 2.25±0.24 |
| Verge grass | 44.8 | 84.6 | 43.1 | nd[a] | 1.98±0.18 |
| Horticultural waste | 21.8±0.3 | 79.7±1.3 | 25.6±2.1 | 0.80±0.07 | 1.29±0.16 |
| MSW-OF | 38.9±3.3 | 53.0±6.2 | 30.3±6.0 | 1.07±0.06 | 7.61±3.99 |
| Group B | | | | | |
| MSW-RDF | 41.4±3.5 | 78.9±0.9 | 68.9±4.3 | 1.08±0.09 | 0.4 |
| Papermill sludge | 49.9±3.0 | 44.2±0.8 | 39.6±0.9 | 0.69±0.09 | 0.14±0.02 |
| Papermill pulp | 24.0±2.0 | 91.1±0.9 | 90.2±0.9 | nd | nd |
| Cellulose | 93.9±0.1 | 99.7±0.1 | 98.5 | 1.22±0.02 | nd |
| Group C | | | | | |
| Bagasse | 94.9±0.1 | 97.1±0.2 | 82.9±0.9 | 1.26±0.06 | 1.43±0.16 |
| Barley straw | 92.7±0.1 | 92.2±0.1 | 80.3±0.9 | 1.27±0.14 | 0.98±0.03 |
| Coffee pulp | 93.3±0.1 | 92.1±2.3 | 56.1±0.5 | 1.25±0.05 | 1.49±0.08 |
| SMC | 38.0±0.7 | 45.0±0.9 | 30.1±2.4 | 0.79±0.04 | 1.16±0.05 |

a not determined

during steady state performance as described previously (Gijzen et al, 1986). Desired hydraulic (HRT) and solid retention times (SRT) were established by adjustable pumps for the supply of fermentation medium and removal of filtered effluent. Homogeneous reactor effluent was removed manually once every day, just before feeding. Samples of 10 ml for determination of pH volatile fatty acids (VFA) and ciliate numbers were removed from the fermenters every other day 4 h after feeding. Operating conditions of HRT, SRT and loading rates during the various experiments are listed in Table 2.

## Analytical procedures

Neutral detergent fibre (NDF), acid detergent fibre (ADF), cellulose (C), hemicellulose (HC) and lignin (L) (Permanganate method)

TABLE 2. Experimental conditions

| Substrate | Pre-treatment[a] | SRT (h) | HRT (h) | LR[b] ($gVS.l^{-1}.d^{-1}$) | alfalfa ($gVS.l^{-1}.d^{-1}$) | EP[c] days | FM[d] |
|---|---|---|---|---|---|---|---|
| Group A | | | | | | | |
| VAW | R 20 | 60 | 15±1 | 24.0 | 3 | 14 | A |
| Alfalfa | - | 60 | 13±1 | 30.4 | - | 21 | A |
| Verge grass | d,R 3 | 60 | 11±1 | 28.9 | - | 14 | A |
| Horticultural waste | d,R 20 | 60 | 16±2 | 21.4 | 3 | 14 | A |
| MSW-OF | d,R 5 | 60 | 12±1 | 30.0 | - | 14 | A |
| Group B | | | | | | | |
| MSW-RDF | d,R 20 | 90 | 12±1 | 20.2 | 3 | 26 | B |
| Papermill sludge | - | 40 | 11±2 | 29.4 | 3 | 14 | B |
| Papermill pulp | d | 60 | 13±1 | 18.5 | 3 | 14 | B |
| Cellulose | R 10 | 60 | 13±1 | 15.0 | 2 | 27 | B |
| Group C | | | | | | | |
| Bagasse | d,R 2 | 60 | 12±1 | 18.3 | - | 23 | B |
| Barley straw | d,R 2 | 60 | 12±1 | 18.3 | - | 14 | B |
| Coffee pulp | d,R 2 | 60 | 12±1 | 17.1 | - | 14 | B |
| SMC | d,R 2 | 60 | 12±1 | 14.3 | 3 | 14 | B |

a d = drying
R n = reduction to less than n mm
b LR = loading rate
c EP = experimental period
d FM = fermentation medium; A = according to Rufener et al (1963);
B = as A but supplemented with $NH_4Cl$ and trace elements

were estimated according to Goering and van Soest (1970). Cell solubles (CS) were defined as volatile solids (VS) minus NDF (Chandler et al, 1980). Total nitrogen (N) content of the substrates was estimated by the modified sulphuric acid-hydrogen peroxide method, according to Ekpete and Cornfield (1964). Analyses of total solids (TS), VS and chemical oxygen demand (COD) were carried out according to standard methods (1975). The extent of VS degradation was estimated by filtering fermenter samples collected at day 8 and 11 and washing the residue three times with the same volume of water (40°C), after which VS content was analysed. Calculation of VS degradation was similar to that of fibre constituents (Gijzen et al, 1986).

Biogas production was measured daily by means of 10 l mariotte flasks containing acidified tap water (approximately 0.02% HCl). Methane content of the biogas was measured according to Hutten et al (1981). Determination of VFA and ciliate numbers and subdivision of ciliates into different groups were as described previously (Gijzen et al, 1986).

## RESULTS

The waste materials used as digester feeds in this study differed markedly from each other with respect to lignin content, the percentage of cell solubles, inorganic material and the C/N ratio (Fig 1, Table 1). On the basis of chemical characteristics the waste materials were subdivided into three groups:

A) Substrates containing a relatively high amount (>40% of VS) of cell solubles (CS-rich substrates),

B) Substrates containing a high percentage of cellulose (> 50% of VS) and a low amount (< 40% of VS) of CS (cellulosic substrates),

C) substrates containing a relatively high amount of lignin (>10% of VS) and a low amount (<40% of VS) of CS (lignocellulosic substrates).

The percentage of inorganic material differed markedly among the substrates, but because of its inert nature, it was not used as a characteristic to differentiate the digester feeds. A high percentage of lignin is usually coupled with a high amount of other cell wall polymers in vegetable matter. This tendency was also found for the waste materials listed in Fig 1. The main difference between group B and C waste materials is the fact that the cellulosic substrates (group B) have been partially or completely delignified in a pulpmill process.

Waste materials belonging to the group of CS-rich substrates were VAW, verge grass, horticultural waste and the organic fraction of MSW (MSW-OF). The cellulosic wastes include papermill sludge, the RDF fraction of MSW (MSW-RDF) and papermill pulp whereas the remaining waste materials from Fig 1 can be classified as lignocellulosic wastes. The model substrates, alfalfa hay and filter paper cellulose,

belong to the groups of CS-rich and cellulosic substrates, respectively. As shown in Table 1, most of the CS-rich substrates contained more nitrogen than the cellulosic and lignocellulosic substrates. A possible effect of nitrogen depletion during the degradation of cellulosic and lignocellulosic substrates was prevented by addition of $NH_4Cl$ to the fermentation medium (Table 2).

Degradation of the substrates was tested under comparable conditions of SRT and HRT, whereas loading rates varied from 17.3 to 32.4 $gVS.l^{-1}.d^{-1}$ among different experiments (Table 2). Although fermentation periods usually were only 14 days, these periods were relatively long as expressed on the basis of fermenter volume turnovers: a fermentation period of 14 days consisted of 28 fluid volume turnovers and 5.6 turnovers of homogeneous fermenter contents. These values exceeded those applied during other anaerobic digestion studies (Lequerica et al, 1984; Pfeffer, 1980; van der Vlugt and Rulkens, 1984).

**Fermentation of CS-rich substrates**

The extent of cell wall polymer (NDF) degradation of CS-rich substrates varied between 54 and 77% (Fig 2A). Because of the high CS content of these substrates digestibility was also established on the basis of organic material (VS). For all substrates VS degradation was markedly higher than degradation of NDF, indicating that the CS fraction of plant material is degraded more efficiently than the fraction of cell wall polymers. The degradation efficiencies were highest for VAW. As a result of the high content of easily degradable compounds, an acidification of the fermenter content was observed immediately after the daily supply of this waste material. Fermenter pH decreased from about 7.2 shortly before feeding to approximately 5.6 2 h after feeding. The pH of the substrate itself was adjusted to 7.0 before addition.

The steady state data on pH and fermentation products are listed in Table 3. VFA and biogas production were almost directly proportional to the amount of substrate digested. Average specific VFA and biogas productions varied between 6.6 - 7.6 mmoles and 0.18-0.23 l per g VS digested, respectively (Table 4). Within these ranges, a relatively high VFA production was correlated with a low biogas production, and

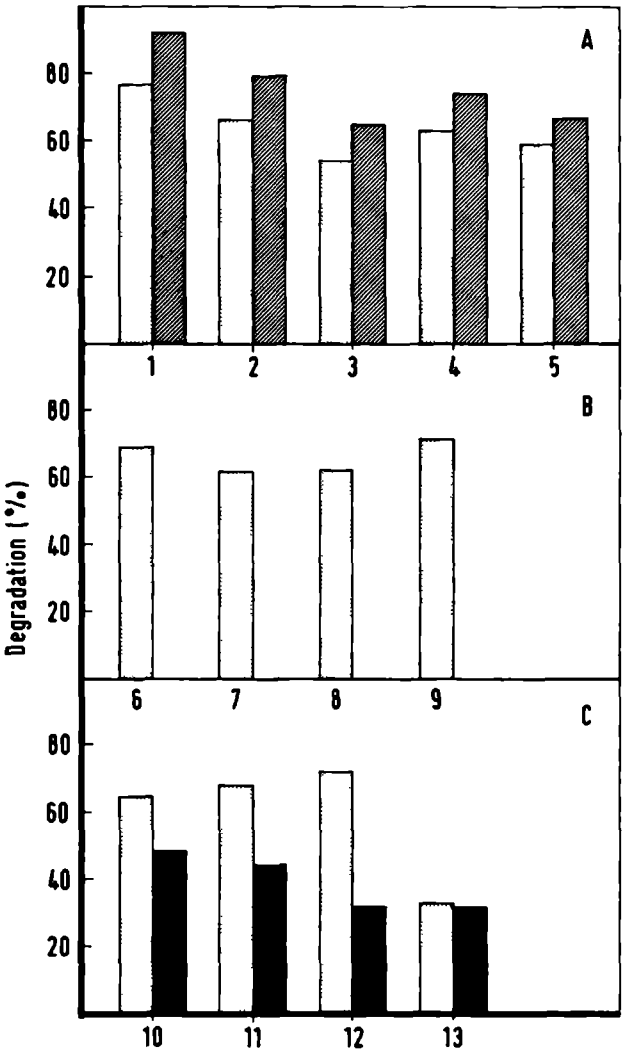



Fig 2. Steady state degradation of NDF (□), VS (▨) and lignin (■) of waste materials belonging to the groups of CS-rich (A; 1=VAW, 2= alfalfa, 3= verge grass, 4= horticultural waste, 5= MSW-OF), cellulosic (B; 6= MSW-RDF, 7= papermill sludge, 8= papermill pulp, 9= filter paper cellulose) and lignocellulosic substrates (C; 10= bagasse, 11= barley straw, 12= coffee pulp, 13= SMC).

TABLE 3. PH and fermentation products (means ± SD) during steady state degradation of various types of digester feed

| Substrate | pH | VFA production[a] mmol.l.$^{-1}$.d$^{-1}$ | molar % A | P | B | Biogas production l.l$^{-1}$.d$^{-1}$ | %$CH_4$ |
|---|---|---|---|---|---|---|---|
| Group A | | | | | | | |
| VAW | 5.6-5.8 | 164 | 59 | 18 | 23 | 5.7±0.5 | nd[b] |
| Alfalfa | 6.3±0.2 | 171±6 | 70 | 18 | 12 | 4.6±0.5 | 42 |
| Verge grass | 6.8±0.2 | 138±1 | 65 | 22 | 13 | 3.9±0.4 | 42 |
| Horticultural waste | 6.6 | 137 | 72 | 15 | 13 | 3.5±0.3 | 40 |
| MSW-OF | 6.7±0.1 | 156 | 59 | 16 | 25 | 3.6±0.1 | 39 |
| Group B | | | | | | | |
| MSW-RDF | 6.5±0.1 | 131±6 | 69 | 23 | 8 | 3.3±0.3 | nd |
| Papermill sludge | 6.3±0.1 | 137 | 67 | 26 | 7 | 5.8±0.5 | 43 |
| Papermill pulp | 6.2±0.2 | 109±9 | 69 | 27 | 4 | 2.6±0.3 | 34 |
| Cellulose | 6.5±0.2 | 95±9 | 70 | 24 | 6 | 2.4±0.7 | 38±2 |
| Group C | | | | | | | |
| Bagasse | 6.9±0.1 | 71±8 | 66 | 25 | 9 | 1.9±0.2 | 36±1 |
| Barley straw | 6.7±0.1 | 101±9 | 74 | 18 | 8 | 2.6±0.2 | 38 |
| Coffee pulp | 6.9±0.2 | 87±13 | 71 | 21 | 8 | 2.2±0.2 | 38±4 |
| SMC | 7.1±0.1 | 25±3 | 61 | 31 | 8 | 0.4±0.1 | 16 |

a A = Acetate, P = Propionate, B = Butyrate
b nd = not determined

conversely.

Protozoa belonging to the *Entodinium* group were predominant in reactors fed with CS-rich substrates (Table 5). The extremely low number of ciliates observed during the fermentation of VAW probably resulted from a temporarily low pH. The fact that the ciliates present during this experiment were almost exclusively representatives of the *Entodinium* group suggests that these species may be less sensitive than other ciliate species to low pH values. Ciliates belonging to the group of Holotrichs were present in high numbers in the inoculum, but were almost completely absent in all reactors during steady-state conditions.

TABLE 4. Specific productions [a] of VFA and biogas (means ±SD) during steady state fermentation of various types of digester feed

| Substrate | VFA production $mmol.g^{-1}VS$ | Biogas production $l.g^{-1}VS$ |
|---|---|---|
| Group A | | |
| VAW | 6.61 | 0.23±0.02 |
| Alfalfa hay | 7.22±0.25 | 0.19±0.02 |
| Verge grass | 7.46±0.05 | 0.21±0.02 |
| Horticultural waste | 7.58 | 0.19±0.02 |
| MSW-OF | 7.60 | 0.18±0.01 |
| Group B | | |
| MSW-RDF | 8.18±0.37 | 0.21±0.02 |
| Papermill sludge | 6.82 | 0.29±0.02 |
| Papermill reject | 8.17±0.67 | 0.20±0.02 |
| Cellulose | 7.87±0.74 | 0.20±0.05 |
| Group C | | |
| Bagasse | 6.06±0.68 | 0.16±0.02 |
| Barley straw | 8.11±0.72 | 0.21±0.02 |
| Coffee pulp | 7.07±1.06 | 0.18±0.02 |
| SMC | 4.37±0.52 | 0.07±0.02 |

a per gVS digested

**Fermentation of cellulosic substrates**

In contrast to the CS-rich substrates, the group of cellulosic materials (group B) contain a high amount of cell wall polymers. Therefore degradation was estimated on the basis of NDF determinations only. Steady-state NDF degradation of the cellulosic substrates were all in the range of 60-70% (Fig 2B).

Specific VFA and biogas productions were calculated to be 6.82-8.18 mmoles and 0.20 - 0.29 l per g VS digested (Table 4). A low specific VFA production in the case of papermill sludge (6.82 $mmol.g^{-1}VS$) correlates with a relatively high biogas production (0.29 $l.g^{-1}VS$). The

TABLE 5. Ciliate numbers and species distribution (means ±SD) during steady state fermentation of various types of digester feed

| Substrate | protozoa $10^3.ml^{-1}$ | % of total | | | |
|---|---|---|---|---|---|
| | | *Diplodinum* | *Epidinum* | *Entodinum* | Holotrichs |
| Group A | | | | | |
| VAW | 16±8 | 6±3 | 2±2 | 92±9 | 0 |
| Alfalfa | 67±5 | 40±10 | 19±2 | 41±4 | 0 |
| Verge grass | 60±7 | 38±5 | 7±1 | 55±7 | 0 |
| Horticultural waste | 73±14 | 14±4 | 23±10 | 60±17 | 3±2 |
| MSW-OF | 42 | 41 | 7 | 51 | 1 |
| Group B | | | | | |
| MSW-RDF | 65±4 | 62±3 | 8±2 | 30±2 | 0 |
| Papermill sludge | nd[a] | | | | |
| Papermill pulp | nd | | | | |
| Cellulose | 65±2 | 82±5 | 4±2 | 14±5 | 0 |
| Group C | | | | | |
| Bagasse | 62±3 | 52±6 | 5±1 | 43±2 | 0 |
| Barley straw | 61±7 | 57±5 | 7±2 | 34±7 | 2±1 |
| Coffee pulp | 67±9 | 55±3 | 5±1 | 40±2 | 0 |
| SMC | nd | | | | |
| Inoculum | 256±159 | 15±6 | 2±1 | 68±5 | 15±4 |

a not determined

molar proportions of individual VFA were rather constant among the various substrates of group B, but differed from CS-rich substrates by a lower yield of butyric acid and an increased production of propionic acid (Table 3). pH values, ranged between 6.3 and 6.5, and were rather stable during a 24 h fermentation period. Obviously the degradation of cellulosic substrates proceeded equable during a 24 h period.

In contrast to the results obtained with CS-rich substrates ciliates of the *Diplodinium* group were predominant in reactors fed with cellulosic substrates (Table 5). Determination of ciliate numbers by means of microscopical enumeration was rather difficult during the degradation of pulpmill sludge, because the size (30 - 150 µm) and shape

of substrate particles and rumen ciliates were in the same range. Protozoal numbers were roughly estimated to be 50-75.$10^3$ cells/ml, with *Diplodinium* as the predominant species.

**Fermentation of lignocellulosic substrates**

NDF degradation efficiencies were relatively high with values ranging between 64-72% except for SMC which was much less degradable (Fig 2C). Despite the relatively low loading rate applied during the digestion of SMC only 32% of NDF was digested. The extent of digestion probably would have been even lower if alfalfa was omitted.

In all experiments performed with lignocellulosic substrates a loss of lignin was observed to an extent of 32-48%. As pointed out previously the loss of lignin might be attributed to solubilization of low molecular lignin during the degradation of other cell wall polymers (Gijzen et al, 1986). Consequently, real NDF fermentation based on the degradation of cellulose and hemicellulose will be somewhat lower as shown in Fig 2C. Real NDF degradation of SMC however was significantly lower, because this waste material contains equal amounts of cellulose and lignin which were almost equally well degraded (35 and 31%, respectively). As a consequence of the apparent lignin degradation, also the specific production of VFA and biogas during the fermentation of SMC were lower as compared to other lignocellulosic wastes (Table 4). The molar proportions of individual VFA observed at the fermentation of lignocellulosic substrates were comparable to those determined with cellulosic substrates (Table 3). Average pH values were somewhat higher as compared to experiments performed with CS-rich and cellulosic substrates. This might have resulted from the lower loading rate applied during the degradation of lignocellulosic waste materials.

The number and species composition of ciliates in reactors fed with lignocellulosic waste materials were comparable to those observed with cellulosic substrates (Table 5). Ciliate numbers were not determined during the experiment with SMC, because the high content of anorganic material strongly interfered in the microscopical enumeration.

The economical feasibility of anaerobic digestion of solid organic waste strongly depends on the rate of the process. Recently we reported the complete decomposition of filter paper cellulose at a rate of 25.8 $gVS.l^{-1}.d^{-1}$ by use of a novel two-phase process, referred to as 'rumen derived anaerobic digestion' (RUDAD-) process (Gijzen et al, 1987). The results of the present study demonstrate that the acidogenic phase of this system can be applied for the efficient degradation of various waste materials. Conversion rates up to 25 $g.l^{-1}.d^{-1}$ for CS-rich wastes and 20 $g.l^{-1}.d^{-1}$ for cellulosic wastes obtained in the artificial rumen digester were considerably higher than those reported in other anaerobic digestion studies. Loading rates applied in this study were about 10 to 20 times as high as those reported for similar waste materials such as fruit and vegetable wastes (Knol et al, 1978), domestic refuse (Pfeffer, 1980; van der Vlugt and Rulkens, 1984; Pathe et al, 1982), papermill sludge (Takeshita et al, 1981) rice straw (Lequerica et al, 1984), SMC (Baader et al, 1981) and coffee pulp (Calzada et al, 1981).

A subdivision of waste materials was made on the basis of their lignin, cellulose and CS content because the relative amounts of these compounds generally determine digestibility. CS-rich waste materials may be considered as readily digestible substrates, because the CS fraction includes easily degradable compounds such as starch, pectin and soluble sugards. This may explain the relatively high degradation efficiencies obtained with VAW, alfalfa and horticultural waste. Lignin on the other hand has been generally recognized to limit the extent of cell wall carbohydrate degradation. Chandler et al (1980) estimated that lignin protects about 2.4 times its own weight of cell wall carbohydrates from microbial degradation. A severely depressed NDF degradation was observed for SMC which contained 37% lignin (% of VS). However degradation efficiency still was markedly higher than values reported by Baader et al (1981) who obtained virtually no degradation of SMC. The high degradation efficiencies obtained for other lignocellulosic wastes were probably due to the relatively low lignin content together with the low loading rates applied.

Major differences were observed in fermentation products and ciliate species composition during degradation of different substrates. Ciliates belonging to the *Entodinium* group were predominant in the inoculum and in reactors fed CS-rich substrates, whereas the *Diplodinium* group was predominant during the fermentation of cellulosic and lignocellulosic substrates. These results correspond with the fact that many *Entodinium* species require starch for growth in vitro (Coleman, 1980), whereas high levels of cellulase activity have been demonstrated in *Diplodinia* (Orpin, 1984). The relative proportions of individual VFA were markedly affected by substrate composition. During the fermentation of CS-rich substrates the molar proportion of butyrate was significantly higher than with cellulosic and lignocellulosic substrates. On the other hand propionate production was higher for (ligno-)cellulosic substrates. Although Erfle et al (1982) reported an effect of pH on molar proportions of individual VFA, no direct correlation between pH and VFA distribution was observed in this study.

All experiments in this study were performed at an arbitrarily chosen loading rate and at fixed retention times for solids and liquids in the reactors. Degradation efficiencies might be further optimized by well-chosen conditions of SRT, HRT and loading rate.

This study deals with the acidogenesis from solid organic wastes in an artificial rumen digester. As a result the main fermentation products consist of VFA and only relatively small amounts of biogas. An overall conversion of organic matter into biogas may be accomplished by a serial connection of the acidogenic reactor to a high-rate methane reactor as was reported previously for pure cellulose (Gijzen et al, 1987). As a consequence of the possible high loading rate and the short retention time needed, only relatively small reactor volumes are required in the application of this system.

## REFERENCES

Anonymous (1975) Standard Methods for the examination of water and waste water. Thrd ed American Public Health Association, New York

Baader W, Schuchardt F, Grabbe K (1981) Gewinnung van biogas aus organischen rückständen. Der Champignon 236: 26-32

Calzada JF, de Léon OR, de Arriola MC, de Micheo F, Rolz C, de Léon R, Menchú JF (1981) Biogas from coffee pulp. Biotechnol Lett 3:

713-716
Chandler JA, Jewell WJ, Gossett JM, van Soest PJ, Robertson JB (1980) Predicting methane fermentation biodegradability. Biotechnol Bioeng Symp 10: 93-107
Coleman GS (1980) Rumen ciliate protozoa. Adv Parasitol 18: 121-174
Cowling EB, Kirk TK (1976) Properties of cellulose and lignocellulosic materials as substrates for enzymic conversion processes. Biotechnol Bioeng Symp 6: 95-123
Demeyer DI (1981) Rumen microbes and digestion of plant cell walls. Agric Environ 6: 295-337
Ekpete DM, Cornfield AH (1964) Comparison of Kjeldahl and sulphuric acid - hydrogen peroxide methods and their salicylic acid - thiosulphate modifications for determining total nitrogen in grasses. Analyst 89: 670-673
Erfle JD, Boila RJ, Teather RM, Mahadevan S, Sauer FB (1982) Effect of pH on fermentation characteristics and protein degradation by rumen microorganisms in vitro. J Dairy Sci 65: 1457-1464
Fahey GG, Jung HG (1983) Lignin as a marker in digestion studies: a review. J Anim Sci 57: 220-225
Ghosh S, Pohland FG (1974) Kinetics of substrate assimilation and product formation in anaerobic digestion. J Water Pollut Cont Fed 46: 748-759
Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986). Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol 25: 155-162
Gijzen HJ, Zwart KB, Verhagen FJM, Vogels GD (1987) High-rate two-phase process for the anaerobic degradation of cellulose, employing rumen microorganisms for an efficient acidogenesis. Submitted for publication
Goering HK and van Soest PJ (1970) Forage fiber analysis. USDA, ARS Agricultural handbook no 379
Hutten TJ, de Jong MH, Peeters BPH, van der Drift C, Vogels GD (1981) Coenzyme M (2-mercapto-ethanesulfonic acid)-derivatives and their effects on methane formation from carbondioxide and methanol by cell-free extracts of Methanosarcina barkeri. J Bacteriol 145: 27-34
Kaspar HF, Wuhrmann K (1978) Kinetic parameters and relative turnover of some important catabolic reactions in digesting sludge. Appl Environ Microbiol 36: 1-7
Knol W, van der Most MM, de Waart J (1978) Biogas production by anaerobic digestion of fruit and vegetable waste. A preliminary study. J Sc Fd Agric 29: 822-830
Ladisch MR, Lin KW, Voloch M, Tsao GT (1983) Process considerations in the enzymatic hydrolysis of biomass. Enzyme Microb Technol 5: 82-101
Lequerica JL, Vallés S, Flors A (1984) Kinetics of rice straw methane fermentation. Appl Microbiol Biotechnol 19: 70-74
Noike T, Endo G, Chang JE, Yaguchi JI, Matsumoto JI (1985) Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion. Biotechnol Bioeng 27: 1482-1489
Orpin CG (1984) The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim Fd Sci Technol 10: 121-143
Pathe PP, Alone BZ, Titus SK, Bhide AD (1982) Studies on parameters

during anaerobic digestion of organic fraction of refuse. Ind J Environ Hlth 24: 8-13
Pfeffer JT (1980) Domestic refuse as a feed for digesters. In: Stafford DA, Wheatly BI, Hughes DE (eds) Anaerobic digestion 1979. Applied science publishers Ltd, London, p 187-203
Rufener WH Jr, Nelson WO, Wolin MJ (1963) Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11: 196-201
Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21: 195-213
Takeshita N, Fujimura E, Mimoto N (1981) Energy recovery by methane fermentation of pulp mill waste water and sludges. Pulp and Paper Canada 82: 99-103
van der Vlugt AJ, Rulkens WH (1984) Biogas production from a domestic waste fraction. In: Ferrero GL, Ferranti MD, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste. Elsevier applied science publishers, London, New York, p 245-250

# ANAEROBIC DIGESTION OF A CELLULOSE FRACTION OF DOMESTIC REFUSE BY MEANS OF RUMEN MICROORGANISMS

Gijzen HJ, Zwart KB, Teunissen MJ, Vogels GD,
(submitted for publication)

## SUMMARY

The anaerobic digestion of a cellulose-enriched fraction of domestic refuse by means of rumen microorganisms in an 'artificial rumen' digester was studied. Various combinations of solids and liquid retention times and loading rates were applied to establish optimum conditions for the acidogenic phase digestion of the refuse fraction. An optimal substrate conversion of about 72% was obtained at a loading rate of 23.4 g of volatile solids per litre per day ($gVS.l^{-1}.d^{-1}$) and a solids retention time of 90 h. Variation of dilution rate between 1.04 and 3.14 fermenter volume turnovers per day had no effect on degradation efficiency. At a loading rate of 23.4 $gVS.l^{-1}.d^{-1}$ a differential removal rate of solids and liquids appeared to be necessary to obtain an effective degradation of the refuse fraction.

## INTRODUCTION

The mineralization of organic material into methane and carbon dioxide by anaerobic microorganisms is becoming an increasingly attractive means of waste treatment and resource recovery (Stafford et al, 1980). The refuse generated in urban areas represents an abundant source of renewable cellulosic biomass for the potential production of biogas (Klass and Ghosh, 1973). The annual production of domestic refuse in Europe amounts to about 275 kg per capita and still continues to increase (de Baere and Verstraete, 1984). Moreover, the costs of current disposal methods like dumping or incineration are increasing rapidly. Anaerobic digestion of domestic refuse could provide an attractive means of reducing disposal costs through the simultaneous reduction of waste volume and recovery of methane as an energy source. Recent reports on the biomethanation of domestic refuse however indicate that the overall reaction rate in conventional, completely mixed reactors is slow, consequently resulting in long residence times and low loading rates of the substrate (Pfeffer, 1974, 1980; Pathe et al, 1982; van der Vlugt and Rulkens, 1984). The rate limitation in these systems is probably caused by the low cellulolytic activities and slow

specific growth rates of the organisms involved (Noike et al, 1985). Therefore the economical feasibility of anaerobic refuse digestion might be strongly enhanced by the development of a process with increased cellulolytic activity.

Recently we proposed the application of rumen microorganisms in an 'artificial rumen' reactor to achieve a more effective digestion of cellulosic residues (Gijzen et al, 1986). Because of their high cellulolytic activity, rumen microorganisms may have considerable potentials to the anaerobic degradation of domestic refuse. The effective decomposition of various solid organic wastes, including domestic refuse, by this acidogenic digestion process has been demonstrated previouslv (Gijzen et al, 1987a). The present paper reports on the results of a study to optimalize the degradation of a cellulosic fraction of domestic refuse by the 'artificial rumen' system . The influence of various combinations of solid (SRT) and liquid retention times (HRT) and loading rates (LR) have been investigated.

## MATERIALS AND METHODS

### Substrate

The substrate for anaerobic digestion used in this study consists of a cellulose-rich waste fraction obtained from a mechanical separation system for municipal solid waste (MSW). The cellulosic fraction forms the residual material of MSW after separation of a fine organic fraction (< 40 mm), metals and large pieces of plastics and paper. The residual fraction, which constitutes approximately 50% of the original MSW, consists mainly of small (< 400 mm) pieces of paper and plastics. Because of its high calorific value this fraction is usually referred to as 'refuse derived fuel' (RDF).

Before using RDF as a feed for anaerobic digestion, synthetic cloths, stones and plastics were carefully separated by hand. The remaining RDF was shredded to a particle size of about 2-3 cm. After drying (48h, 70°C), RDF was used as a digester feed. The chemical composition of RDF is shown in Table 1. The remaining plastics (5.5%) were present as coatings of packings.

Table 1. Chemical composition of the substrates[a]

| Determination | | RDF | Alfalfa |
|---|---|---|---|
| Total solids (TS) | | 41.4±3.5 | 92.9±0.9 |
| Volatile solids | (% of TS) | 83.7±3.4 | 91.5±1.0 |
| NDF | ( " ) | 68.9±3.9 | 45.0±0.4 |
| ADF | ( " ) | 63.1±3.2 | 32.2±0.7 |
| Cellulose | ( " ) | 61.0±2.4 | 23.1±0.3 |
| Hemicellulose | ( " ) | 5.8±1.5 | 12.8±0.7 |
| Lignin | ( " ) | 2.1 | 9.1±0.8 |
| Cell solubles | ( " ) | 14.8±3.6 | 46.5±0.9 |
| Plastics | ( " ) | 5.5 | - |
| Total nitrogen | ( " ) | 0.4 | 2.25±0.24 |
| COD[b] | ($gO_2.g^{-1}TS$) | 1.1±0.1 | 1.1±0.1 |

[a] Means ± standard deviation

[b] Chemical oxygen demand

**Fermentation device**

Acidogenesis from RDF was performed in a 3 l volume 'artificial rumen' reactor operating with differential removal rates of solids and liquids, as was described previously (Gijzen et al, 1986). The reactor was maintained at 39°C. The inoculum consisted of 250 ml strained rumen fluid obtained from a sheep provided with a rumen fistula. The substrate was added once every day and consisted of a desired loading rate of RDF, supplemented with 3.0 $gVS.l^{-1}$ of ground (1-2 mm) alfalfa (van Heeswijk, Veghel, the Netherlands). At the day of inoculation, twice the normal amount of substrate was administered.

Fermentation medium according to Rufener et al (1963) was modifed by the addition of $NH_4Cl$ (28 mM) as a nitrogen source and trace elements (0.2 $ml.l^{-1}$) according to Vishniac and Santer (1957). Desired hydraulic (HRT) and solid retention times (SRT) were established by means of adjustable peristaltic pumps for the supply of fresh fermen-

tation medium and for removal of filtered effluent. Homogeneous reaction mixture was removed once daily just before addition of the substrate. Despite the diurnal variation of the working volume, the average volume over a 24 h period was 1.5 l in all experiments.

Unless otherwise stated, experiments were performed over periods of 14 days, with the first 7 days serving as an acclimatization period. Samples (10 ml) for the determination of pH, volatile fatty acids (VFA) and ciliate numbers were removed from the reactors every other day, 4h after substrate addition.

### Analytical procedures

Neutral detergent fibre (NDF), acid detergent fibre (ADF) cellulose, hemicellulose (HC) and lignin (permenganate method) were determined according to Goering and van Soest (1970). Cell solubles were calculated as volatile solids (VS) minus NDF (Chandler et al, 1980). All other analyses were as described previously (Gijzen et al, 1986, 1987a).

## RESULTS

### Acclimatization time

The acclimatization time of rumen microorganisms to the substrates and operation conditions was established in an experiment over a period of 26 days (equivalent to 52 complete changes of fermentation medium). SRT, HRT and loading rate (LR) were maintained at 90 h, 12 h and 23.4 $gVS.l^{-1}.d^{-1}$ (including 3.0 g VS of alfalfa), respectively. Fibre degradation was determined between day 7 and 10 and between day 21 and 24 after inoculation. Degradation efficiencies for all fibre fractions were only slightly lower during the second period (Table 2). NDF and ADF degradation efficiencies were almost equal which is consistent with the low hemicellulose (HC) content of the substrate (Table 1).

Productions of VFA and biogas showed some minor fluctuations during the first 7 days of operation, but remained rather stable during the

Table 2. Fibre degradation (%)

| Fibre fraction | Period 1 day 7-10 | Period 2 day 21-24 |
|---|---|---|
| NDF | 72 | 67 |
| ADF | 67 | 64 |
| HC | 99 | 88 |

rest of the experimental period, with average (±SD) values of 129±9 $mmol.l^{-1}.d^{-1}$ and 2.7±0.3 $1.l^{-1}.d$, respectively (Fig 1). The molar proportions of individual VFA were 69%, 22%, and 9% for acetate, propionate and butyrate, respectively. Because of the almost constant production of VFA fermenter pH was rather stable at 6.45 during the entire experimental period (Fig 1B). Protozoal numbers and species composition changed markedly during the first 7 days of operation (Fig 1C). During this period protozoal numbers declined from $104.10^3 cells.ml^{-1}$ immediately after inoculation to a steady-state level of $66±5.10^3 cells.ml^{-1}$. The relative number of ciliates belonging to the *Entodinium* group, decreased significantly whereas that of the *Diplodinium* group increased within the first 7 days after inoculation. Holotrichs, which accounted for 12% of the ciliates in the inoculum, disappeared almost completely within the same period. Steady-state ciliate species distribution is in agreemeent with previous observations (Gijzen et al, 1987a).

The results indicate that fermenter performance is stable after an acclimatization period of approximately 7 days.

**Effect of loading rate (LR)**

In order to establish optimum conditions for the degradation of RDF, experiments of 14 days each were performed at various combinations of LR, SRT and dilution rates (D). Within each set of experiments only one of the parameters was varied. Degradation and fermen-

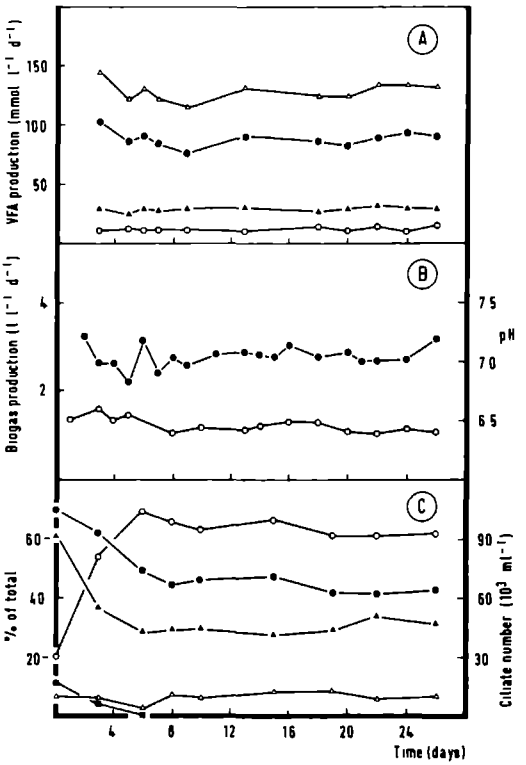



Fig 1. Effect of operation time on fermentation products and ciliates. A, production of acetate(●) propionate (▲) butyrate (o) and total VFA (Δ). B, pH (o) and biogas production (●). C, ciliate numbers (●) and percentages of ciliates belonging to the groups of *Diplodinium* (o), *Entodinium* (▲), *Epidinium* (Δ) and Holotrichs (□).

tation products were determined during steady state (day 7-14).

The effect of LR on degradation efficiency was studied in 3 experiments at 15.2, 19.3 and 23.4 gVS.$l^{-1}$.$d^{-1}$, while SRT and D were adjusted to 60 h and 2.0 fermenter volume turnovers per day (FV.$d^{-1}$), respectively. Degradation efficiency of all fibre fractions was highest at a LR of 19.3 gVS.$l.^{-1}$.$d^{-1}$ (Table 3). HC degradation was almost complete at all loading rates. A higher LR resulted in a decreased fermenter pH. As is shown in Table 4 ciliate numbers were somewhat lower at the highest loading rate applied. The percentage of *Entodinia*

Table 3. Effect of loading rate on fibre degradation and pH during steady state operation

| LR $gVS.l^{-1}.d^{-1}$ | pH | degradation (%) | | |
|---|---|---|---|---|
| | | NDF | ADF | HC |
| 15.2 | 6.85 | 71 | 67 | 99 |
| 19.3 | 6.60 | 76 | 73 | 99 |
| 23.4 | 6.50 | 66 | 62 | 94 |

Table 4. Effect of LR on ciliate numbers and species composition during steady state operation

| LR $gVS.l^{-1}.d^{-1}$ | Ciliates $10^3.ml^{-1}$ | % of total | | |
|---|---|---|---|---|
| | | *Diplodinium* | *Epidinium* | *Entodinium* |
| 15.2 | 78±5 | 74±2 | 8±1 | 18±1 |
| 19.3 | 78±2 | 78±2 | 8±2 | 14±1 |
| 23.4 | 64±10 | 62±7 | 5±3 | 33±3 |

was higher and that of *Diplodinia* was somewhat lower at this loading rate.

Although degradation at a LR of 23.4 g $VS.l^{-1}.d^{-1}$ was still rather efficient, no higher LR was applied, since the consequent higher content of total solids in the fermenter caused serious mixing problems. Therefore further optimization studies with respect to SRT and D variations were performed at a LR of 23.4 $gVS.l^{-1}.d^{-1}$.

**Effect of solid retention time (SRT)**

The effect of SRT on RDF degradation was studied in 6 separate experiments at a constant D and LR of 2.0 $FV.d^{-1}$ and 23.4 $gVS.l^{-1}.d^{-1}$, respectively, whereas SRT was varied between 24 and 144 h. Under these

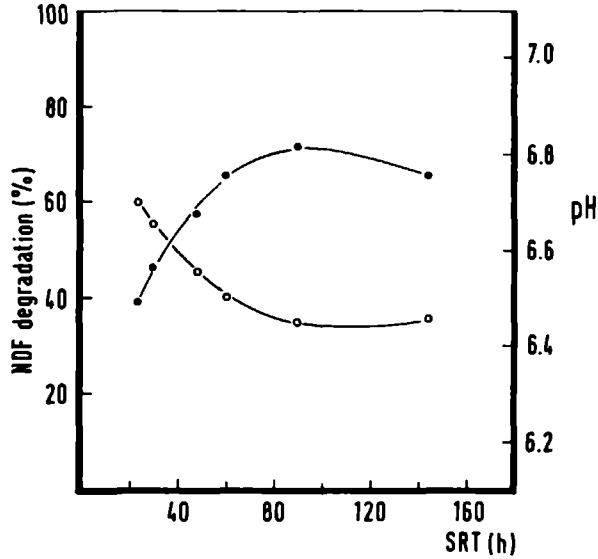



Figure 2. Effect of solid retention time on pH (o) and NDF degradation (●).

conditions optimal substrate degradation was obtained at a SRT of 90 h (Fig 2). A sharp decrease in degradation efficiency was observed at SRT below 60 h. The slightly lower degradation at a SRT of 144 h was probably due to the increased content of total solids which hindered proper mixing of reactor contents. pH decreased slightly at longer SRT which is in agreement with the improved degradation efficiency and the resulting increase of VFA production.

Ciliate numbers were strongly affected by SRT as is shown in Fig 3. The increase in ciliate numbers at longer SRT is probably due to the decreased washout of these organisms. Apart from the absolute number of ciliates, also the species composition was affected by SRT (Fig 4). At shorter SRT the frequency of ciliates belonging to the *Entodinium* group increased, whereas that of the *Diplodinium* group decreased. This suggests that *Entodinia* would be less sensitive to washout, which is in agreement with the relatively short generation times reported for these organisms (Coleman, 1979). Holotrichs were absent in all experiments.

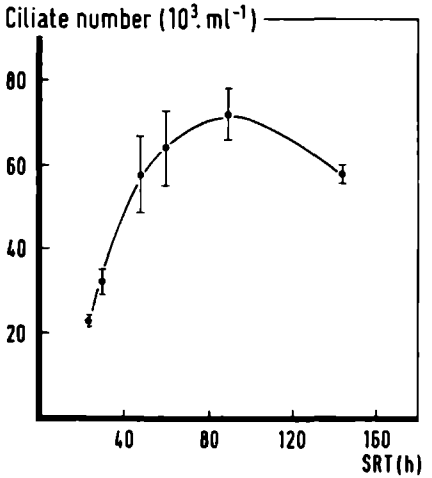



Figure 3. Effect of solid retention time on steady state ciliate numbers (average ±SD)

**Effect of dilution rate (D)**

Because of the differential removal rates of solids and liquids applied in the 'artificial rumen' system the separate effects of SRT and D variation were studied. The effect of D on the fermentation process was established in seven short-term experiments at a SRT of 144 h and a LR of 23.4gVS.$l^{-1}$.$d^{-1}$, whereas D was varied between 0.52 and 3.14 FV.$d^{-1}$. The effect of D on NDF degradation efficiency is given in Fig 5. Except for the lowest dilution rate, NDF degradation was comparably high at all D-values applied. As a result of a decreased removal rate of acid fermentation products pH values of reactor contents dropped at lower dilution rate (Fig 5). The extremely low degradation efficiency observed at a D of 0.52 FV.$d^{-1}$ is probably due to the inhibitory effect of acidification on the fermentation process.

The production of VFA and biogas were correlated with the extent of digestion of the substrate (Fig 6). At D varying between 1.63 and 3.14 FV.$d^{-1}$ average molar proportions of acetate, propionate and butyrate were rather constant at 70, 21 and 9% respectively (Fig 7). At lower D-values, however an increase was observed in the relative proportion

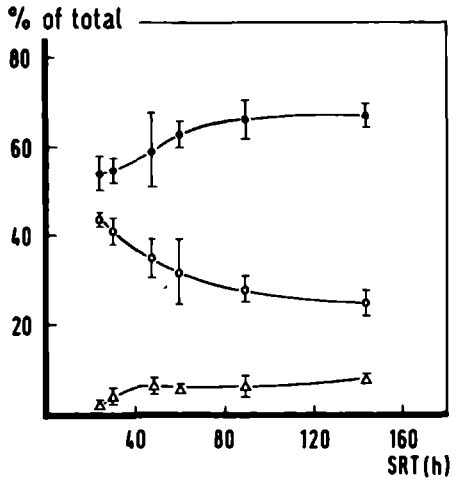



Figure 4. Effect of solid retention time on ciliate species distribution (average ±SD). (●) *Diplodinium* group, (o) *Entodinium* group, (Δ) *Epidinium* group

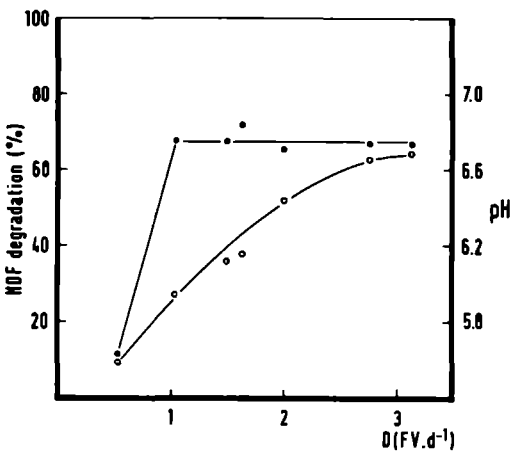



Figure 5. Effect of dilution rate on pH (o) and NDF degradation (●)

of propionate, whereas the proportions of acetate and butyrate decreased. A comparable shift in molar proportions of individual VFA was reported by Erfle et al (1982) on a decrease of the pH of the fermentation medium. Consequently, the observed changes in VFA composition at

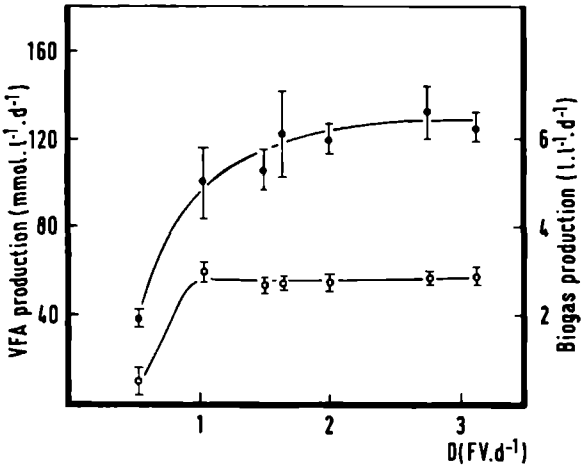



Figure 6. Effect of dilution rate on steady-state productions (average ±SD) of VFA (●) and biogas (o)

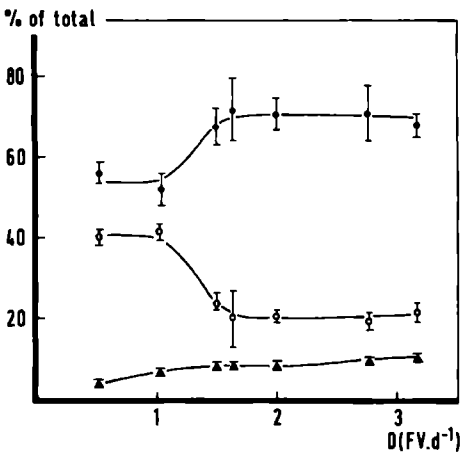



Figure 7. Effect of dilution rate on the molar proportions (average ±SD) of acetate (●), propionate (o) and butyrate (▲)

different D-values may be a result of pH variation.

The effect of D on ciliate numbers and species composition is shown in Fig 8. Ciliate numbers were in the range of 49 - 63.$10^3$ cell.$ml^{-1}$ at all D except at 0.52 FV.$d^{-1}$, when ciliates were completely absent probably as a result of acidification. This is in agreement with the

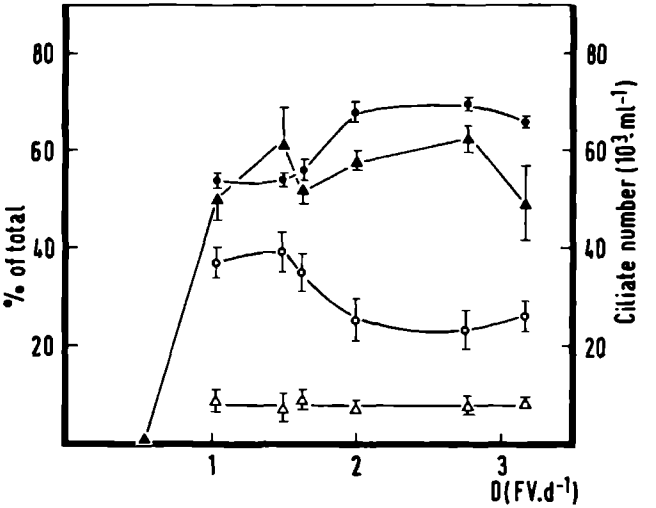



Figure 8. Effect of dilution rate on steady-state ciliate numbers and ciliate species distribution (average ±SD). (●) *Diplodinium* group, (o) *Entodinium* group, (Δ) *Epidinium* group

results of a previous study with grass as a substrate in which protozoal numbers were markedly affected by D-variation at pH values below 6.0 (Gijzen et al, 1986). On lowering D below 2.0 FV.d$^{-1}$ the relative number of *Entodinia* increased, whereas the proportion of ciliates belonging to the *Diplodinium* group decreased. The shift in protozoal composition may be attributed to a selective washout of small *Entodinium* species through the filter. The results of experiments at various dilution rates indicate that there is no real optimum for D, but fluid removal rate should be high enough to prevent acidification of the fermenter contents.

**Digestion at equal SRT and HRT**

In all previous experiments SRT was kept markedly longer than HRT by the application of a filter unit in the reactors. This mode of operation was derived from the in vivo situation of the rumen (Weller and Pilgrim, 1974) and was found necessary during in vitro fermentation of a grass-grain mixture (Gijzen et al, 1986). However, from Fig 2 and 5 it appears that SRT and D values as low as 24 h and 1.0 FV.d$^{-1}$

can be applied during digestion of RDF. This suggests that also a combination of a SRT of 24 h and a D of 1.0 $FV.d^{-1}$ should be possible, which means that retention times of solids and liquids are adjusted equally. To test this mode of operation, an experiment was carried out at a LR of 23.4 $gVS.l^{-1}.d^{-1}$ and solids and liquids residence time were adjusted to 36 h. In this setup the filter became superfluous and was removed from the fermenter. Table 5 summerizes the steady state data on NDF digestion and product formation. Fermentation characteristics were comparable to data obtained at a SRT of 30 h and D of 2.0 $FV.d^{-1}$. Because of the accumulation of VFA, pH dropped to 6.0.

### Omission of alfalfa

RDF represents a rather poor substrate for microbial degradation since it consists almost exclusively of carbohydrates. Therefore, a small amount of alafalfa was added as an additional source of nutrients in all experiments. To study the effect of alfalfa omission, two expe-

Table 5. Steady state data (average ±SD) during the fermentation of RDF at equal residence times for solids and liquids

| Determination | Average |
|---|---|
| NDF digestion | 45 |
| VFA production ($mmol.l^{-1}.d^{-1}$) | 87±8 |
| Molar %, acetate | 69 |
| propionate | 25 |
| butyrate | 6 |
| pH | 6.0 |
| Biogas production ($l.l^{-1}.d^{-1}$) | 1.0±0.4 |
| Ciliates ($10^3.ml^{-1}$) | 35±6 |

Table 6. Effect of alfalfa omission on steady state fermenter performance

| Determination | + alfalfa | - alfalfa |
|---|---|---|
| NDF degradation (%) | 72 | 71 |
| VFA production ($mmol.l^{-1}.d^{-1}$) | 123±9 | 126±10 |
| Molar %, acetate | 68 | 69 |
| propionate | 23 | 23 |
| butyrate | 9 | 8 |
| pH | 6.45 | 6.50 |
| Biogas production ($l.l^{-1}.d^{-1}$) | 73±9 | 70±8 |
| Ciliates ($10^3.ml^{-1}$) | 2.6±0.3 | 2.6±0.2 |

riments were performed over a period of 35 days, one with RDF (20.4 $gVS.l^{-1}.d^{-1}$) and alfalfa (3.0 $gVS.l^{-1}.d^{-1}$) and one with RDF only (23.3 $gVS.l^{-1}.d^{-1}$). SRT and dilution rate were 90 h and 2.0 $FV.d^{-1}$, respectively.

Steady-state NDF degradation and productions of VFA and biogas were almost similar in both experiments (Table 6). Furthermore ciliate numbers in both experiments were almost identical. The data suggest that under the conditions applied in this study alfalfa is not needed as an additional nutrient source during digestion of RDF.

## DISCUSSION

In a complex multistep process the kinetic characteristics of the slowest step govern the overall conversion rate. If refractory organic wastes such as domestic refuse are used as substrates for anaerobic digestion, the hydrolysis step has shown to be rate limiting (Pfeffer, 1974; Boone, 1982; Noike et al, 1985). The results of the present study demonstrate that the rate of acidogenesis from RDF may be very high if rumen microorganisms are applied. The 'artificial rumen'

reactor was operated at a loading rate of 23.4 $gVS.l^{-1}.d^{-1}$, which is about 10 to 20 times as high as loading rates reported for conventional reactors treating domestic refuse (Pfeffer, 1974; Pfeffer, 1980; van der Vlugt and Rulkens, 1984; Pathe et al, 1982). The low loading rates in these systems result from the relatively long SRT needed (20-40 days) to achieve effective anaerobic decomposition. In contrast, substrate residence times in the rumen amount to 1-2 days only (Hungate 1966). Similar short substrate residence times could be applied in the 'artificial rumen' digester, but degradation efficiency dropped markedly at SRT below 60 h.

Another major characteristic of the in vivo system is the fact that fluid turnover rate is much higher than that of the solid substrate (Weller and Pilgrim, 1974). During the degradation of RDF, fluid removal rate should be at 1.0 $FV.d^{-1}$ or higher in order to maintain a high degradation efficiency. The relatively high D is probably required to prevent acidification of fermenter contents which would result in decreased fibre degradation (Erfle et al, 1982; Hoover et al, 1984; Gijzen et al, 1986). This may explain the inefficient degradation observed at a D of 0.52 $FV.d^{-1}$ where the pH was low. Operation of a digester at equal residence times of solids and liquids might be beneficial for an industrial application of the process, because in this case the expensive separation system of solids and liquids becomes redundant. Such conditions could be applied in this study and a removal rate of 0.67 $FV.d^{-1}$ for both solids and liquids did not result in acidification of fermenter contents. However, digestion was limited by the low substrate retention. Therefore differential removal rates of solids and liquids appear to be required to realize an effective and stable acidogenic digestion of RDF. Optimal substrate conversion was obtained at a LR of 23.4 $gVS.l^{-1}.d^{-1}$ and a SRT of 90 h, whereas variation of D between 1.04 and 3.14 $FV.d^{-1}$ had no effect on the digestion process.

In conclusion, the results presented in this study demonstrate that the application of rumen microorganisms in a rumen-like digester might be useful to improve acidogenic digestion of refractory organic waste materials such as domestic refuse. A complete conversion of RDF into $CH_4$ and $CO_2$ may be achieved by the conversion of VFA in a subsequent high-rate methane reactor as was described previously for pure cellu-

lose as a substrate (Gijzen et al, 1987b).

## REFERENCES


Boone DR (1982) Terminal reactions in the anaerobic digestion of animal waste. Appl Environ Microbiol 43: 57-64

Chandler JA, Jewell WJ, Gossett JM, van Soest PJ, Robertson JB (1980) Predicting methane fermentation biodegradability. Biotechnol Bioeng Symp 10: 93-107

Coleman GS (1979) Rumen ciliate protozoa. In: Levandowsky M, Hutner SH (eds) Biochemistry and physiology of protozoa, 2. Acad Press, New York, p381-408

de Baere L, Verstraete W (1984) Anaerobic fermentation of semi-solid and solid substrates. In: Ferrero GL, Ferranti MP, Naveau H (eds) Anaerobic digestion and carbohydrate Hydrolysis of waste. Elsevier Applied Science Publishers, London, New York, p195-208

Erfle JD, Boila RJ, Teather RM, Mahadevan S, Sauer FD (1982) Effect of pH on fermentation characteristics and protein degradation by rumen microorganisms in vitro. J dairy Sci 65: 1457-1464

Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986) Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol 25: 155-162

Gijzen HJ, Lubberding HJ, Verhagen FJ, Zwart KB, Vogels GD (1987a) Application of rumen microorganisms for an enhanced anaerobic degradation of solid organic waste materials. Agric Wastes, submitted

Gijzen HJ, Zwart KB, Verhagen FJ, Vogels GD (1987b) High-rate two-phase process for the anaerobic degradation of cellulose, employing rumen microorganisms for an efficient acidogenesis. Biotechnol Bioeng, submitted

Goering HK, van Soest PJ (1970) Forage Fiber analysis. USDA, ARS Agric Handbook No. 379

Hoover WH, Kincaid CR, Varga GA, Thayne WV, Junkins L Jr (1984) Effects of solids and liquids flows on fermentation in continuous cultures. IV pH and dilution rate. J Anim Sci 58: 692-700

Hungate RE (1966) The rumen and its microbes. Acad Press, New York

Klass DL, Ghosh S (1973) Fuel gas from organic wastes. Chem Technol Nov, 689-698

Noike T, Endo G, Ghang JE, Yaguchi JI, Matsumoto JI (1985) Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion. Biotechnol Bioeng 27: 1482-1489

Pathe PP, Alone BZ, Titus SK, Bhide AD (1982) Studies on parameters during anaerobic digestion of organic fraction of refuse. Indian J Environ HLTh 24: 8-13

Pfeffer JT (1974) Temperature effects on anaerobic fermentation of domestic refuse. Biotechnol Bioeng 16: 771-787

Pfeffer JT (1980) Domestic refuse as a feed for digesters. In: Stafford DA, Wheatly BI, Hughes DE (eds) Anaerobic digestion 1979. Applied science Publishers Ltd., London, p187-203

Rufener WH Jr, Nelson WO, Wolin MJ (1963) Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11:

196-201
Stafford DA, Hawkes DL, Horton R (1980) Methane production from waste organic matter, CRC Press BOCA Raton, Florida, USA
van der Vlugt AJ, Rulkens WH (1984) Biogas production from a domestic waste fraction. In: Ferrero GL, Ferranti MD, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste. Elsevier Applied Science Publishers, London, New York, p245-250
Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21: 195-213
Weller RA, Pilgrim AF (1974) Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Br J Nutr 32: 341-352

CHAPTER 7

# ANAEROBIC DIGESTION OF A CELLULOSIC FRACTION OF DOMESTIC REFUSE BY A TWO-PHASE RUMEN-DERIVED PROCESS

Zwart KB, Gijzen HJ, Cox P, Vogels GD,
(submitted for publication)

## SUMMARY

The anaerobic degradation of a cellulosic fraction of domestic refuse by a rumen-derived two-phase process was studied. Because of their high cellulase activities rumen microorganisms were used as an inoculum in the acidogenic phase. The stability of the two-phase digestion process was established in two long-term experiments of 80 and 420 days, respectively. High loading rates up to 27.5 $g.l^{-1}.d^{-1}$ in combination with a solid retention time of only 4 days resulted in a stable degradation efficiency of about 60%. After a prolonged period of operation, part of the VFA catabolizing activities of the methanogenic phase had been taken over by the acidogenic phase.

## INTRODUCTION

Anaerobic digestion of solid organic wastes into biogas is of current interest in terms of waste volume reduction and recovery of renewable energy (Stafford et al, 1980). However, a large-scale application of this process to industrial and municipal solid wastes has been hindered by the low rates of substrate conversion and biogas production.

In a recent series of papers we demonstrated that the use of rumen microorganisms in an 'artificial rumen' digester resulted in an enhanced conversion efficiency of cellulosic substrates (Gijzen et al, 1986; 1987a, c). One of the waste materials tested was a cellulosic fraction of domestic refuse, usually referred to as 'refuse derived fuel' (RDF) (Gijzen et al, 1987a). The conditions for optimal degradation of RDF have been established previously by means of short-term experiments (Gijzen et al, 1987b). However, for a succesfull application of the rumen-derived anaerobic digestion (RUDAD-) process it is necessary to demonstrate its stability over prolonged periods of operation. Especially with RDF as a substrate, long-term experiments may provide important information on the stability of the RUDAD-process, because of the presence of possibly hazardous compounds in domestic refuse.

## Two-phase digestion process

The principles of the two-phase reactor system used, have been described previously (Gijzen et al, 1987c). The acidogenic reactor (RI), containing rumen microorganisms consisted of a 30 l volume double-walled glass construction (20 l working volume) with a PVC bottom and top. The top contained a lid (16 cm diameter) for the removal of reactor contents and adddition of substrate and several tubes for the passage of fluids and biogas. Dissolved reaction products and minute dispersed particles were continuously removed from RI by means of a variable peristatic pump via a cylindrical filter (height 18 cm, diameter 5 cm, pore size 300 µm) and subsequently fed to a UASB-type methane reactor (RII). The filter could be removed from RI via the lid. The contents of RI were mixed during 1 minute at intervals of 15 minutes by means of a eccentrically placed mechanical stirrer with two propellers. Heavy particles in the RI filtrate were allowed to settle down in a 2 l erlenmeyer in order to prevent clogging of the RII inlet. The precipitated contents were removed once a week.

RII was a 12 l double-walled glass reactor filled with 3.5 l granular sludge obtained from a full-scale UASB-plant (AVEBE de Krim, The Netherlands). Effluent from RII was recirculated into RI via a water seal. Both reactors were kept at 39°C by a thermostatically controlled circulation bath.

## Substrate

Refuse derived fuel (RDF) obtained from a mechanical seapration system for municipal solid waste (VAM, Wijster, the Netherlands) was used as a substrate for the RUDAD-process. Pretreatment and composition of the substrate were as described previously (Gijzen et al, 1987b). The RDF was shredded to a particle size of 5-10 cm and added to RI at desired loading rates. As an additional nutrient source ground (1-2 mm) alfalfa (Van Heeswijk, Veghel, the Netherlands) was

added at a fixed loading rate of 2.5 g total solids per litre per day (g $TS.l^{-1}.d^{-1}$).

## Experimental conditions

LONG-TERM EXPERIMENTS

Two long-term experiments were performed in order to test the stability of the RUDAD-process. Within these experiments tests were performed to measure the effects of an increased loading rate, a decreased fluid recirculation rate through the system and a disconnection of RII resulting in a completely mixed digestion in RI. Experiment 1, which lasted 80 days, was started by inoculating RI with 500 ml fresh strained rumen fluid obtained from a fistulated sheep. The reactor contained 19.5 l fermentation medium (Rufener et al, 1963) supplemented with $NH_4Cl$ (28 mM) as a nitrogen source and trace elements (0.2 $ml.l^{-1}$) according to Vishniac and Santer (1957). The solid retention times (SRT) and loading rates were adjusted as indicated in Table 1 for 5 periods of operation. During period 4 RII was disconnected from RI.

Experiment 2 lasted for more than one year and was started similarly to experiment 1. The sludge which remained in RII at the end of experiment 1 was used again in experiment 2. The characteristics of the four periods of experiment 2 are indicated in Table 2.

SRT was adjusted as follows: each day prior to feeding an appropriate volume of homogeneous contents were removed from RI. During experiment 1 this volume was replaced by fresh fermentation medium to a final volume of 20 l. During experiment 2 the removed contents were sucked through 300 µm pore-size nylon gauge and the filtrate was transferred to RI, whereas the residue was used for analytical procedures.

VFA CONVERSION IN RI

In order to investigate the capacity of VFA conversion in RI 100-ml serum bottles were filled with 25 ml filtered reactor contents and 20 ml fermentation medium. After the addition of acetate (final concentration 42 mM), propionate (14 mM) and butyrate (10 mM) (total volume 5 ml) and 2 ml of reducing solution according to Goering and van Soest (1970) the bottles were sealed, evaporated and filled with $N_2/CO_2$ (80/20, v/v) at 0.3 atm. The bottles were incubated at 39°C in a

rotary shaker (200 rpm). The disappearance of VFA and the concomitant production of methane was followed during 3 weeks. In control incubations 5 ml water instead of VFA was added.

### Analytical methods

Biogas production from RI and from RII in experiment 1 was measured by 25 l mariotte flasks containing acidified tap water (about 0.02% HCl). Since the volume of the mariotte flasks was much smaller than the volume of biogas produced during a 24-h period an electronic gas measurement device was constructed according to the system described by Moletta and Albagnac (1982) for the continuous measurement of biogas from RI and RII. This system is based on the repeated expulsion and registration of a calibrated volume of fluid. The flask containing two level probes was calibrated at 1000 ml. All other analyses were as described previously (Gijzen et al, 1986, 1987c).

## RESULTS

Table 1 presents the results of RDF-degradation during experiment 1. During the two-phase operation degradation efficiency of volatile solids (VS) was 69 and 71% at a loading rate of 17.5 and 22.5 g $TS.l^{-1}.d^{-1}$, respectively. Disconnection of RII (period 4) resulted in a decrease of degradation (61% of VS) despite of the low loading rate of 11.3 $g.l^{-1}.d^{-1}$. The concentration of volatile fatty acids (VFA) in RI and RII was measured 3 times a week. The concentrations of individual VFA in RI are presented in Fig 1. The concentration of acetate fluctuated during period 1 and decreased gradually during periods 2 and 3. A similar pattern was observed for propionate. Butyrate concentration was always lower than 3 mM (not shown) except for period 4 when it increased to 10 mM (Fig 1). Acetate and butyrate conversion in RII were almost complete (85-95%) during periods 1, 2, 3 and 5. Propionate conversion during period 1 was about 60% and increased to 80-90% during periods 2, 3 and 5. The decrease of VFA concentration in RI during periods 2 and 3 was accompanied by an increase in the produc-

Table 1. Experimental conditions, VS degradation, biogas production and protozoal numbers during long-term digestion of RDF (experiment 1)

| Period | days | LR[a] | SRT | D[b] | VS degradation | Biogas production[c] | | Protozoal number |
|---|---|---|---|---|---|---|---|---|
| | | $g.l^{-1}.d^{-1}$ | d | $FV.d^{-1}$ | % | $l.d^{-1}$ | | $10^3.ml^{-1}$ |
| | | | | | | RI | RII | |
| 1 | 0-17 | 17.5 | 7 | 2 | nd[d] | nd | nd | nd |
| 2 | 17-46 | 17.5 | 4 | 2 | 69 | 62±20 | 65±7 | 37 |
| 3 | 46-54 | 22.5 | 4 | 2 | 71 | 80±16 | 70±7 | 59 |
| 4 | 54-72 | 11.3 | 4 | 0.25 | 61 | 46±7 | - | 35 |
| 5 | 72-80 | 11.3 | 4 | 2 | nd | nd | nd | nd |

a LR = loading rate
b D = fluid removal rate from RI
c mean ±SD
d nd = not determined

tion of biogas (Table 1). This was probably due to the establishment of non-flocculant bacteria from RII in RI. Epifluorescence microscopical examination (Doddema and Vogels, 1978) revealed the presence of large numbers of *Methanosarcina* species in RI during period 3. These acetoclastic organisms are not found in high numbers in sheep rumen, whereas they were abundant in the UASB sludge in RII.

In order to study the dynamics of VFA conversion by RI contents, batch-wise incubations were performed with a mixture of acetate, propionate and butyate. The results of a typical incubation are illustrated in Fig 2. Acetate and butyrate disappeared within 10-12 days. The shoulder in acetate disappearance from day 5 to 9 results from the simultaneous conversion of butyrate into acetate. The concentration of propionate, however, remained unchanged until all acetate and butyrate had been converted into biogas.

These results together with the observations in RI described above, clearly demonstrate that a substantial part of the VFA produced in RI was already converted into biogas in the same reactor. Because of the VFA catabolizing activity in RI, a single-phase mode of operation was tested by disconnecting RII (period 4). The disconnection, however, had drastic effects on VFA concentrations in RI (Fig 1). Propionate concentration increased to about 40 mM within 10 day after disconnec-

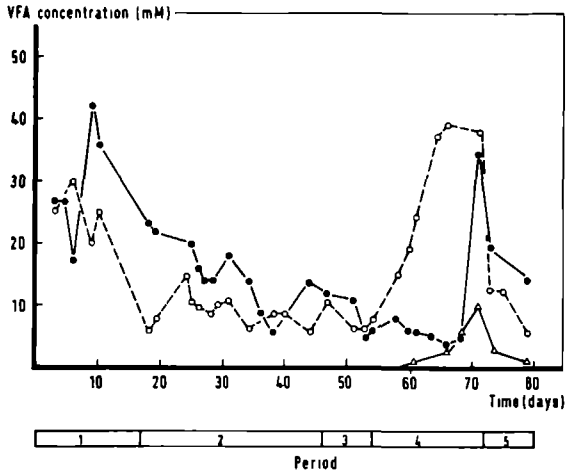



Figure 1. Concentrations of acetate (●), propionate (o) and butyrate (Δ) in the acidogenic phase (RI) during long-term digestion of RDF (experiment 1)

ting RII resulting in a decrease of pH from 7.3 to 6.7 (not shown). Concentrations of acetate and butyrate increased from about 12 days after the disconnection resulting in a further decrease of pH to 5.6. Coupling of RI and RII (period 5) immediately restored the situation prior to the disconnection.

Fig 3 shows the results of RDF degradation during experiment 2. For convenience the results of each period (Table 2) will be discussed separately.

PERIOD 1 (WEEK 0-15)

During period 1 VS and TS degradations were rather constant at approximately 65 and 55%, respectively (Fig 3A). Acetate production decreased whereas propionate production remained constant at 15-20 $mmol.l^{-1}.d^{-1}$ (Fig 3C). Butyrate production was also constant at a value of about 5 $mmoles.l^{-1}.d^{-1}$ (not shown). Biogas production of RI was 70 $l.d^{-1}$ with a methane content increasing from about 20% (week 1-5) to 35% (week 10-15). The overall increase of methane production is in agreement with the decrease of VFA production in RI. Biogas

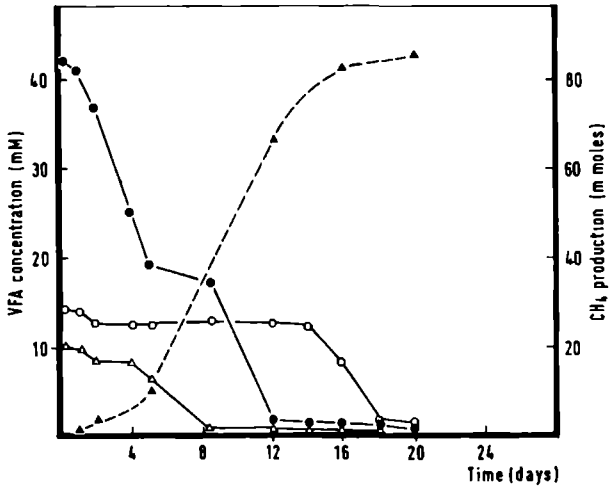



Figure 2. Conversion of a mixture of acetate (●), propionate (○) and butyrate (Δ) during batch-wise incubation with acidogenic phase (RI) reactor contents, removed during period 2 of experiment 1.

production of RII varied somewhat due to inaccurate measurement caused by gas leakage. The methane content of biogas from RII was about 70% (Table 2). In RII 90-95% of VFA from RI were converted (results not shown). The total ciliate number in RI increased from $0.5.10^4$ to $3.5.10^4$ cells.ml$^{-1}$ within three weeks after inoculation. The ciliate species composition changed after inoculation comparable to changes described for short-term experiments (Gijzen et al 1987a).

PERIOD 2 (WEEK 15-34)

The increase of loading rate from 22.5 to 27.5 g.l$^{-1}$.d$^{-1}$ had no effect on degradation efficiency (Fig 3A). The decrease in acetate production in RI, which started in period 1, continues during period 2. However, it is important to note that the VFA 'production' presented in Fig 3C results from the combined action of acidogenic and VFA-catabolizing organism in RI. Propionate production increased, but not as an immediate response to the increase in loading rate (Fig 3C). Biogas production of RI increased immediately after the increase in loading rate (Fig 3B). Although the total biogas production did not

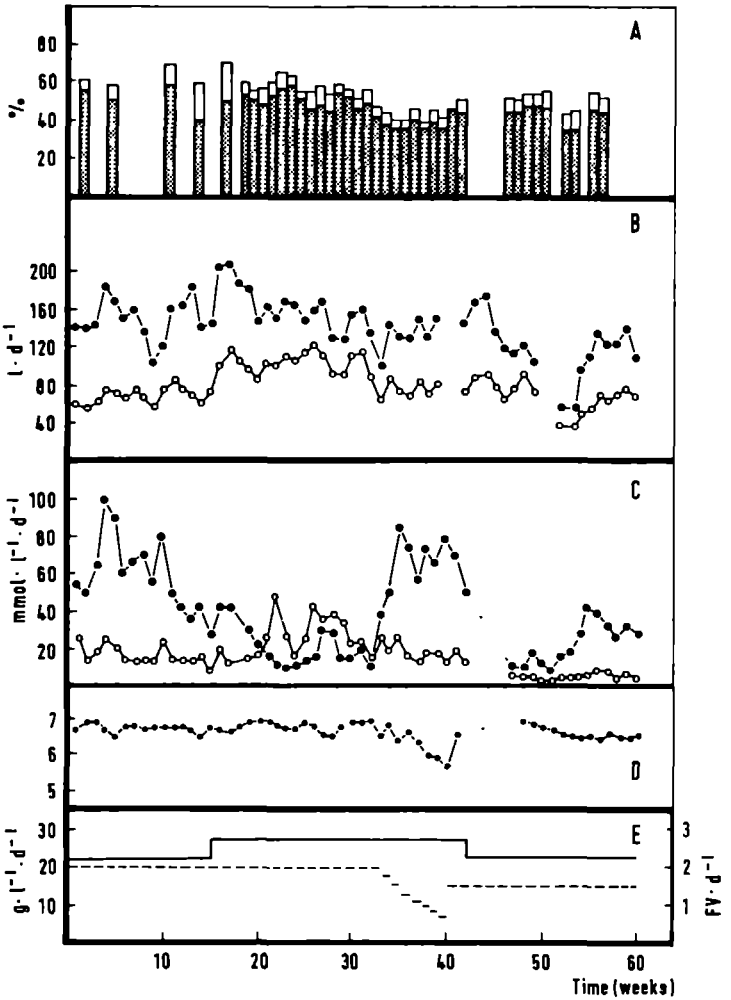



Figure 3. Performance of the RUDAD-process during long-term digestion of RDF (experiment 2). A, degradation of VS ( □ ) and TS ( ▨ ); B, biogas production of RI (o) and RI+RII (●); C, production of acetate (●) and propionate (o); D, pH; E, loading rate (——) and fluid removal rate from RI (---). The dotted intervals in graphs B, C and D represent periods in which no samples were analyzed.

Table 2. Experimental conditions, protozoal numbers and methane content of the biogas from RI and RII during long-term digestion of RDF (experiment 2)

| Period | Weeks | LR[a] $g.l^{-1}.d^{-1}$ | SRT d | D[b] $FV.d^{-1}$ | Protozoal number[c] $10^3.ml^{-1}$ | $CH_4$ content[c] % RI | % RII |
|---|---|---|---|---|---|---|---|
| 1 | 0-15 | 22.5 | 4 | 2 | 5 35 | 27±5 | 71±4 |
| 2 | 15-34 | 27.5 | 4 | 2 | 37±3 | 48±4 | 74±6 |
| 3 | 34-40 | 27.5 | 4 | 2 0.9 | 40±8 | 37±8 | 77±3 |
| 4 | 40-60 | 22.5 | 4 | 1.5 | nd[d] | 49±8 | 73±7 |

a LR = loading rate
b D = fluid removal rate from RI
c mean ±SD
d nd = not determined

increase at the higher loading rate, overall methane production increased as a result of the higher methane content of the biogas from RI (Table 2). Ciliate numbers (Table 2) and pH (Fig 3D) remained unchanged.

PERIOD 3 (WEEK 34-40)

The stepwise decrease in fluid removal rate from RI from 2.0 to 0.9 $FV.d^{-1}$ during period 3 resulted in a decrease of VS degradation from about 60 to 40% (Fig 3A). Because of the lower rate of VFA removal the pH of RI decreased from 7 to 5.7 after which it was decided to terminate this part of the experiment by increasing the fluid recirculation in order to prevent complete reactor failure due to acidification. Surprisingly acetate production of RI increased drastically whereas propionate production was rather stable (Fig 3C). The biogas production of RI decreased slightly whereas the total production from RI and RII remained almost unchanged (Fig 3B). However, $CH_4$ production in RI decreased from 52 to 29 $l.d^{-1}$ whereas that of RII increased from 32 to 48 $l.d^{-1}$. The efficiency of VFA conversion into biogas in RII was not altered by fluid recirculation rate.

PERIOD 4 (WEEK 40-60)

Increase of the fluid circulation to 1.5 $FV.d^{-1}$ and decrease of

loading rate from 27.5 to 22.5 $g.l^{-1}.d^{-1}$ resulted in a rapid increase of pH and restoration of RDF degradation efficiency and VFA and biogas productions to values comparable to those of periods 1 and 2. The low biogas production of weeks 48-55 were due to leakage of the gas measurement device.

PROBLEMS DURING LONG-TERM OPERATION

During both long-term experiments several problems were encountered which are not specific for the RUDAD-process, but are likely to occur in any two-phase degradation system for solid waste materials. Clogging of the filter in RI and of the RII inlet occurred several times. For instance, during the first 25 weeks of experiment 2 clogging at these locations was observed 38 times. Moreover, proper mixing of RI, immediately after feeding at the highest loading rate, was problematic. Occasionally technical failure of the circulation bath or peristaltic pump caused disturbances in reactor temperature and dilution rate.

During experiment 2 biogas production dropped two times rather drastically, in week 20 and 33. This was accompanied by a high viscosity of RI contents 24 h after feeding. The viscosity was normally very low at that time. Apparently degradation was suddenly inhibited, possibly by hazardous components in the substrate. Simply by lowering loading rate for 2 or 3 days and addition of 5-10 l fresh fermentation medium degradation efficiency and biogas production restored to normal levels. Thus, the stability of the RUDAD-process was high enough to overcome the problems mentioned, and reinoculation of RI or RII was not needed during the entire experimental period. However, these problems may explain for a large part the variability in the obtained results.

## DISCUSSION

RDF degradation by the RUDAD-process was demonstrated to be efficient and stable. The VS degradation of approximately 60% at a loading rate of 27.5 $g.l^{-1}.d^{-1}$ was comparable to earlier results obtained in a 1.5 l reactor (Gijzen et al 1987b). The loading rates applied in this

study however are 10 to 20 times as high as those reported for conventional completely mixed reactors treating domestic refuse (Pfeffer 1974, 1980; Pathe et al 1982; van de Vlugt and Rulkens, 1984). Disturbances of the reactor conditions caused by technical trouble or by intent and resulting in a decreased degradation were easily overcome by resetting the original conditions.

A small inoculum of rumen microorganisms of 0.025 reactor volume was sufficient to create ciliate numbers in RI almost comparable to those in the rumen (Hungate, 1966). Even during an experiment lasting for more than one year it was not necessary to reinoculate the reactor. However, as reported earlier (Gijzen et al 1987a) the species composition changed markedly probably due to substrate characteristics. The high stability and rapid growth of a small inoculum of rumen microorganisms are a prerequisite for a large-scale application of the RUDAD-process. Another requirement is an efficiently working separation system between solids and liquids present in RI. A filter system was used in the experiments described in this study. Although filtration was possible on a 30 l scale, it may not be applicable on a large scale because of the increasing volume occupied by the filter. This problem may be overcome by the development of a regenerable filter system or by the application of other methods available for the separation of solids and liquids (Stanton, 1980).

After some time of operation part of the activity of the methanogenic reactor was taken over by the acidogenic reactor. Apparently VFA catabolizing bacteria including acetoclastic methanogens were established in RI after prolonged operation. This is in agreement with the results of a previous study on the two-phase digestion of pure cellulose (Gijzen et al, 1987c). Propionate conversion in RI was less pronounced than that of acetate and butyrate, which is probably due to the fact that its conversion is thermodynamically less favourable (Zehnder and Koch, 1983). The partial conversion of VFA allows for a lowering of the fluid removal rate from RI which would be beneficial for large-scale application of the RUDAD-process. However, the results indicate that fluid removal rate should be high enough to prevent acidification of the reactor content, eventually resulting in reactor failure.

In conclusion, the results presented in this study demonstrate that

the application of the RUDAD-process results in a high-rate digestion of cellulosic wastes such as domestic refuse. Moreover, the process was demonstrated to be stable over extended periods of time which is a prerequisite for an industrial application of the system.

## REFERENCES


Doddema HJ and Vogels GD (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. Appl Environ Microbiol 36: 752-754

Gijzen HJ, Lubberding HJ, Verhagen FJ, Zwart KB, Vogels GD (1987a) Application of rumen microorganisms for an enhanced anaerobic degradation of solid organic waste materials. Agric wastes, submitted

Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986) Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol 25: 155-162

Gijzen HJ, Zwart KB, Teunissen MJ, Vogels GD (1987b) Anaerobic digestion of a cellulose fraction of domestic refuse by means of rumen microorganisms. Biotechnol Bioeng, submitted

Gijzen HJ, Zwart KB, Verhagen FJ, Vogels GD (1987c) High-rate two-phase process for the anaerobic degradation of cellulose, employing rumen microorganisms for an efficient acidogenesis. Biotechnol Bioeng, submitted

Goering HK, van Soest PJ (1970) Forage fiber analysis. USDA, ARS Agric Handbook no. 379

Hungate RE (1966) The rumen and its microbes. Acad Press, New York

Moletta R, Albagnac G (1982) A gas meter for low rates of gas flow: application to the methane fermentation. Biotechnol Lett 4: 319-322

Pathe PP, Alone BZ, Titus SK, Bhide AD (1982) Studies on parameters during anaerobic digestion of organic fraction of refuse. Indian J Environ HLTH 24: 8-13

Pfeffer JT (1974) Temperature effects on anaerobic fermentation of domestic refuse. Biotechnol Bioeng 16: 771-787

Pfeffer JT (1980) Domestic refuse as a feed for digesters. In: Stafford DA, Wheatly BI, Hughes DE (eds) Anaerobic digestion 1979. Appl Sci Publ Ltd, London, p187-203

Rufener WH Jr, Nelson WO, Wolin MJ (1963) Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11: 196-201

Stafford DA, Hawkes DL, Horton R (1980) Methane production from waste organic matter. CRC Press BOCA Raton, Florida, USA

Stanton MJ (1980) Sludge-handling problems and solid/liquid separation. In: Stafford DA, Wheatly BI, Hughes DE (eds) Anaerobic digestion 1979. Appl Sci Publ Ltd, London, p395-407

van der Vlugt AJ, Rulkens WH (1984) Biogas production from a domestic waste fraction. In: Ferrero GL, Ferranti MD, Naveau H (eds) Anaerobic digestion and carbohydrate hydrolysis of waste.

Elsevier Appl Sci Publ, London, New York, p245-250
Vishniac W, Santer M (1957) The thiobacilli. Bact Rev 21: 195-213
Zehnder AJB, Koch ME (1983) Thermodynamic and kinetic interactions of the final steps in anaerobic digestion. In: Proc Eur Symp Anaerobic Waste Water Treatment, Noordwijkerhout, the Netherlands p 86-96

CHAPTER 8

# ANAEROBIC DEGRADATION OF PAPERMILL SLUDGE IN A TWO-PHASE DIGESTER CONTAINING RUMEN MICROORGANISMS AND COLONIZED POLYURETHANE FOAM

Gijzen HJ, Schoenmakers T, Caerteling G, Vogels GD,
(submitted for publication)

## SUMMARY

The use of reticulated polyurethane foam as a support material for the immobilization of methanogenic associations and its application to the anaerobic treatment of fine particulate solid wastes was investigated. The colonization of polyurethane support particles in a continuous upflow reactor fed on a mixture of acetate, propionate and butyrate, was both rapid and dense. The combination of rumen microorganisms and colonized support particles in a two-phase digester resulted in an efficient anaerobic decomposition of papermill sludge.

## INTRODUCTION

The catabolism of volatile fatty acids (VFA) and acetoclastic methane formation have been identified as rate-limiting steps in the anaerobic digestion of soluble wastes (Ghosh and Pohland, 1974; Lawrence and McCarty, 1969). The limitation is caused by the low specific growth rates of the bacteria involved in these conversions. Consequently, long retention times are required to prevent washout of the active biomass in conventional, completely mixed reactors.

Several high-rate anaerobic digesters, based on the retention of slowly growing populations, have been developed in the past 15 years (van den Berg, 1984). One of the methods to increase biomass retention is to immobilize methanogenic consortia on inert support materials. The application of reticulated polyurethane foam as a support material has been reported previously (Fynn and Whitmore, 1982; Huysman et al, 1983) and was shown to result in a rapid and dense colonization.

Applications of support materials have been restricted mainly to the treatment of waste waters (van den Berg, 1984). This study deals with the colonization of reticulated polyurethane foam and its utilization in the anaerobic digestion of fine particulate organic waste. Recently we demonstrated that a high-rate acidogenesis of cellulosic waste materials could be obtained by the use of rumen microorganisms in an 'artificial rumen' digester (Gijzen et al, 1987a). This paper

reports on the combination of rumen microorganisms and colonized polyurethane support particles in a two-phase digester treating papermill sludge.

## MATERIALS AND METHODS

### Immobilization of methanogenic associations

Reticulated polyurethane foam (density 18 kg/m³; 20 pores per linear cm) was used as a support material for the immobilization of methanogenic consortia. A 10 l volume cylindrical upflow reactor (15.5 cm internal diameter) was filled with 10 $g.l^{-1}$ of polyurethane support material, used in cubes with an average edge size of 2.2 cm. The reactor was maintained at 39°C. The packed reactor was subsequently filled with half strength buffer solution according to Rufener et al (1963), supplemented with trace elements (0.1 $ml.l^{-1}$) according to Vishniac and Santer (1957) and $NH_4Cl$ (14 mM). The reactor was inoculated with 300 ml crushed granular sludge, obtained from a full scale UASB-plant (AVEBE, de Krim). The same medium as mentioned above, but supplemented with yeast extract (0.2 $g.l^{-1}$), sodium acetate (38 mM), propionate (12.8 mM) and butyrate (8.7 mM), was continuously fed to the upflow reactor at a rate of 2.6 reactor volume turnovers per day. This corresponds to a hydraulic retention time of 9.2 h. After 5 days of operation, remaining sludge was removed from the bottom of the reactor. The progress of immobilization was monitored by determination of VFA conversion.

### Two-phase reactor design

A two-phase reactor, consisting of an acidogenic and a methanogenic phase, was developed. A 25 l reactor (39°C) was inoculated with 800 ml of fresh strained rumen fluid which was obtained from a fistulated sheep. The reactor was subsequently filled to a volume of 5 l with pre-heated (39°C) buffer solution according to Rufener (1963), modified by the addition of $NH_4Cl$ (28mM) and trace elements (0.2 $ml.l^{-1}$).

The day after inoculation, colonized support particles were transferred from the upflow reactor into the two-phase reactor, resulting in a total working volume of 15 l. The digester feed consisted of partially dehydrated papermill sludge, supplemented with a small amount of ground alfalfa. The substrate was added once daily except for the day of inoculation when twice the normal loading rate was applied. The composition of the substrates is shown in Table 1. The lower part of the reactor (acidogenic phase) was stirred and homogeneous content was continuously pumped over the upper layer of support particles (methanogenic phase) at a rate of 25 $l.d^{-1}$. Every day 2.5 l of homogeneous acidogenic phase content was replaced by an equal volume of fresh fermentation medium with suspended substrate. A schematic diagram of the two-phase reactor is presented in Fig 1.

**Sampling and analyses**

Samples for determination of pH, VFA and neutral detergent fibre (NDF) were taken from the daily homogeneous reactor effluent. Biogas production was monitored daily and methane content of the biogas was determined in triplo twice every week. Analytical methods were as described previously (Gijzen et al, 1986, 1987b).

Table 1. Composition of the substrates[a]

| Determination | Alfalfa | Papermill sludge |
|---|---|---|
| Dry weight (dw)% | 92.9±0.7 | 40.4±0.2 |
| VS[b] ( % of dw) | 90.7±1.6 | 38.2±0.5 |
| NDF[c] (% of dw) | 46.0±1.8 | 32.2±0.7 |
| Total N ( % of dw) | 2.3±0.2 | 0.14±0.02 |
| COD[d] ($gO_2.g^{-1}dw$) | 1.08±0.04 | 0.69±0.09 |

a Average ±SD

b Volatile solids

c Neutral detergent fibre (Goering and van Soest, 1970)

d Chemical oxygen demand

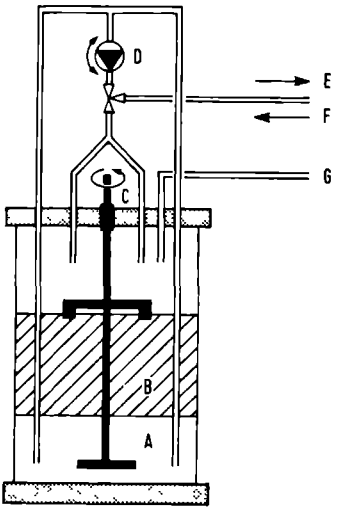



Figure 1. Schematic representation of the two-phase reactor. A, acidogenic phase; B, methanogenic phase; C, stirrer; D, bidirectional sludge pump; E, homogeneous effluent removal; F, feeding and addition of fresh fermentation medium. G, gas outlet.

## RESULTS AND DISCUSSION

### Colonization of polyurethane

Colonization of the polyurethane support particles as assessed by VFA conversion proceeded at a high rate (Fig 2). Within 13 days after inoculation, stable conversion efficiencies of 80-90% and 95-100% were obtained for acetate and butyrate, respectively. Optimal propionate degradation of about 80% was reached after 20 days of operation. The rate of colonization was somewhat lower as compared to previous reports on studies with polyurethane (Fynn and Whitmore, 1982; Huysman et al, 1983). The differences in colonization rate may be accounted for by differences in the substrates applied. After 3 weeks of colonization biomass concentration amounted to 0.02 ± 0.003 g VS per $cm^3$

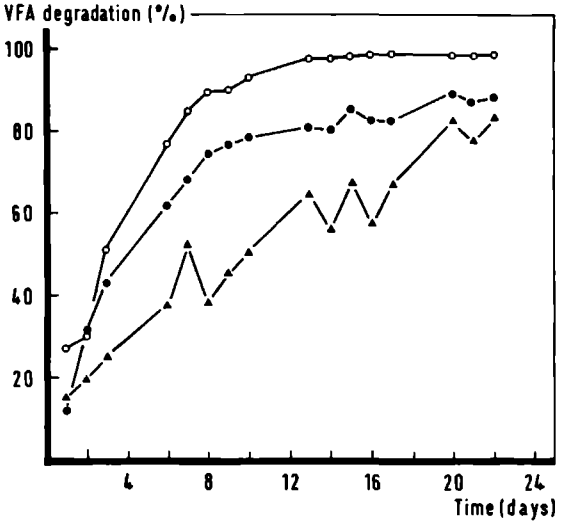



Figure 2. Degradation efficiency of acetate (●) propionate (▲) and butyrate (O) during the colonization of polyurethane support particles.

of support material.

Scanning electron microscopic examination of the colonized support particles revealed the presence of aggregates of bacteria in the cavities of the polyurethane foam (Fig 3). In contrast to previous observations on the colonization of polyurethane (Fynn and Whitmore, 1982) the bacteria appeared to be physically associated with the support material. The biofilm consisted mainly of long filamentous *Methanotrix*-type bacteria and smaller numbers of *Methanosarcina* species.

**Two-phase reactor performance**

After about 3 weeks of colonization, the support particles were transferred into the two-phase reactor, which was inoculated with rumen microorganisms one day before. Substrate loading rates were 75 g VS.$d^{-1}$ during the first 30 days of operation and 102 g VS.$d^{-1}$ thereafter, including 21 g VS of alfalfa at both loading rates. Performance of the two-phase digester during a 60 days period of operation is sum-

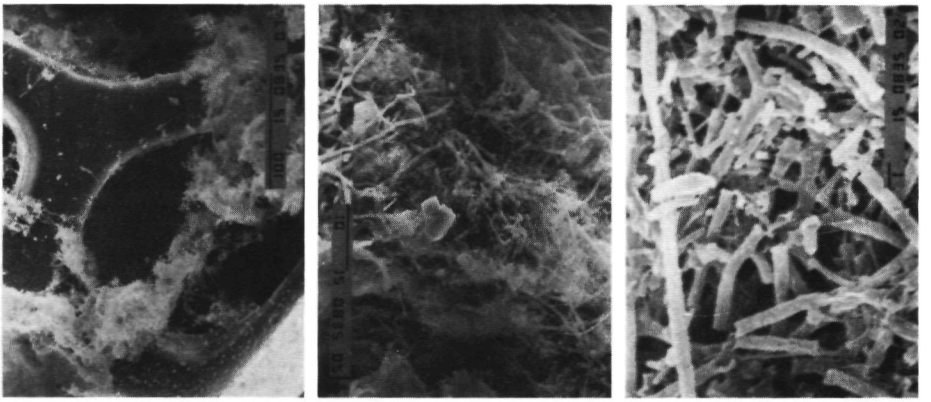


Figure 3. Scanning electron micrographs of colonized polyurethane foam.

marized in Fig 4.

From day 5 to 30 after inoculation, biogas production was rather stable at 33±3 $l.d^{-1}$. Methane content of the biogas amounted to 55±4% during the entire experimental period, except for the first 5 days (Fig 4A). The higher loading rate applied after 30 days of operation resulted in an increase of biogas production to 42±2 $l.d^{-1}$.

Methane yields obtained were 0.24 and 0.23 $l.g^{-1}$VS added, at loading rates of 75 and 102 g $VS.d^{-1}$, respectively. From these methane yields and by assuming the organic part of the substrate to be $(C_6H_{10}O_5)_n$, degradation efficiencies were calculated to be 55 and 52%, respectively. However, NDF degradations determined as the difference between NDF content of the substrate and that of the homogeneous effluent was much higher than those expected on the basis of methane formation (results not shown). NDF degradation decreased from about 75% during the first 20 days to about 60-65% at the end of the test period. The differences between expected and measured NDF degradation efficiencies may be accounted for by accumulation of substrate particles in the polyurethane layer, by incomplete conversion of substrates to methane and by removal of VFA via the homogeneous effluent. Therefore, real NDF degradation probably will be in the range

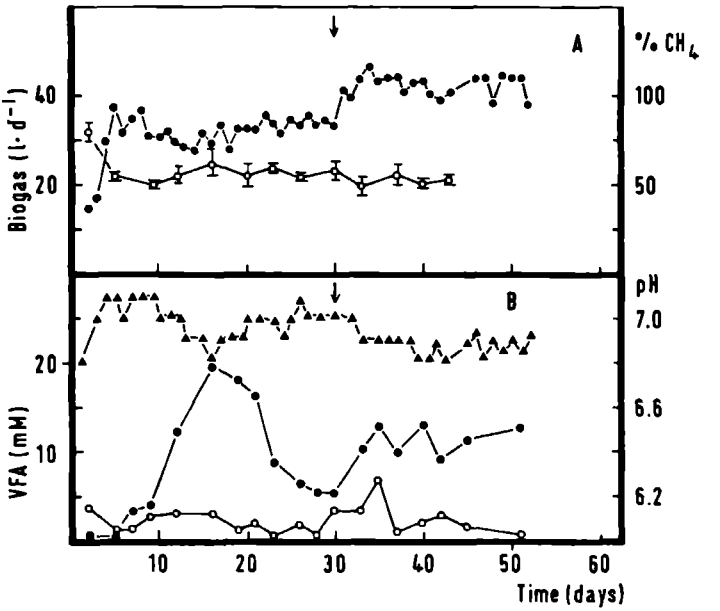



Figure 4. Performance of a two-phase digester fed on papermill sludge. A, biogas production (●), methane content (o); B, pH (▲), concentration of acetate (o) and propionate (●). Arrow indicates the increase of loading rate.

of 55-65%. These values are comparable to NDF decomposition, previously reported for the acidogenic phase digestion of papermill sludge by a mixed population of rumen microorganisms (Gijzen et al, 1987a). However, in the present experimental set-up no filtration system for the separation of solids and liquids was required, which makes the design much more attractive for an industrial application of the process. In spite of the high content of anorganic material of the papermill sludge used in this study, loading rates and conversion efficiencies were higher than those reported by Takeshita et al (1981).

Concentrations of individual VFA in the acidogenic phase showed some fluctuations untill a stable level was maintained from day 35 on (Fig 4B). The fluctuations were most explicit for the concentrations of propionate which showed a temporary increase to a maximal level of 20 mM between day 12 and 21 and a second increase after day 30 to a

stable level of about 10 to 13 mM. A similar pattern was observed for acetate, but concentrations were markedly lower (< 7 mM) and were almost zero after 45 days of operation. The concentration of butyrate (not shown) did not exceed the 1 mM level during the entire test period. The observed fluctuations in VFA levels reflect disturbances in the balance between their production in the acidogenic phase and their subsequent degradation in the methanogenic phase. Specific accumulation of propionate may be explained by the fact that its anaerobic conversion is thermodynamically less favourable than that of acetate or butyrate (Zehnder and Koch, 1983). The increased concentration of propionate observed between day 12 and 21 may be a result of washout of loosely attached biomass from the polyurethane support particles and the higher propionate level after day 30 probably originates from the higher loading rate applied. The pH values in the acidogenic phase were correlated with fluctuations in the concentrations of VFA and varied between 6.8 and 7.1 (Fig 4B).

Rumen ciliates were present in high numbers (about $50.10^3$ cells.$ml^{-1}$) during the entire experimental period. The exact microscopical enumeration of ciliates was hindered by substrate particles which had a similar size and shape.

In conclusion the results described in this study demonstrate that polyurethane may be used as an excellent support material for slowly growing methanogenic phase bacteria. The combination of rumen microorganisms in the acidogenic phase and colonized support particles in the methanogenic phase of a two-phase reactor resulted in a high-rate anaerobic digestion of papermill sludge. Further work is required to optimize the two-phase digester performance and to establish its potential application to other particulate solid waste materials.

## REFERENCES

Fynn GH, Whitmore TN (1982) Colonization of polyurethane reticulated foam biomass support particle by methanogen species. Biotechnol Lett 4: 577-582

Ghosh S, Pohland FG (1974) kinetics of substrate assimilation and product formation in anaerobic digestion. J Water Pollut Control Fed 46: 748-759

Gijzen HJ, Lubberding HJ, Verhagen FJ, Zwart KB, Vogels GD (1987a)

Application of rumen microorganisms for an enhanced anaerobic degradation of solid organic waste materials. Agric Wastes, submitted

Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986) Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol 25: 155-162

Gijzen HJ, Zwart KB, Verhagen FJ, Vogels GD (1987b) High-rate two-phase process for the anaerobic degradation of cellulose, employing rumen microorganisms for an efficient acidogenesis. Biotechnol Bioeng, submitted

Goering HK, van Soest PJ (1970) Forage fibre analysis. USDA, ARS Agricultural Handbook No. 379

Huysman P, van Meenen P, van Assche P, Verstraete W (1983) Factors affecting the colonization of non porous and porous packing materials in model upflow methane reactors. In: Proc Eur Symp Anaerobic Waste Water Treatment, Noordwijkerhout, the Netherlands, p187-200

Lawrence AW, McCarty PC (1969) kinetics of methane fermentation in anaerobic treatment. J Water Pollut Control Fed 42: 1-17

Rufener WH Jr, Nelson WO, Wolin MJ (1963) Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11: 196-201

Takeshita N, Fujimura E, Mimoto N (1981) Energy recovery by methane fermentation of pulp mill waste water and sludges. Pulp Pap Can 82: 171-175

van den Berg L (1984) Developments in methanogenesis from industrial waste water. Can J Microbiol 30: 975-990

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21: 195-213

Zehnder AJB, Koch ME (1983) Thermodynamic and kinetic interactions of the final steps in anaerobic digestion. In: Proc Eur Symp Anaerobic Waste Water Treatment, Noordwijkerhout, the Netherlands, p86-96

# SUMMARY / SAMENVATTING

Worldwide photosynthetic fixation of $CO_2$ is estimated to yield up to 15 to $20.10^{10}$ of dry plant material annually. The mineralization of cellulosic biomass proceeds almost exclusively by microbial processes, occurring both in aerobic and anaerobic environments. During the anaerobic mineralization organic materials are converted into methane and carbon dioxide (biogas). The process occurs wherever organic matter accumulates and oxygen becomes depleted such as in aquatic sediments, marshes, paddy fields, but also in the digestive tract of herbivorous animals.

Anaerobic digestion has been applied to the treatment of sewage sludge since the end of the previous century. Anaerobic waste water treatment has made great progress recently by the development of several high-rate digesters based on the concept of immobilisation of the slowly growing microbial biomass. In contrast, anaerobic digestion of solid organic waste has not been very efficient in terms of loading rates, solid residence times and extent of decomposition, and therefore it has been limited mainly to animal wastes and secondary waste water sludge. The low conversion rates are generally ascribed to the low rate of hydrolysis of cellulosic biomass. The feasibility of anaerobic digestion of cellulosic wastes might be strongly enhanced by the application of microorganisms exhibiting high cellulase activities.

This thesis describes the development of a novel two-stage anaerobic digestion process, applying a unique microbial population from the forestomach of ruminants. The rumen may be considered as a high-rate natural digester for the conversion of cellulosic substrates. Ruminants and all other herbivorous animals have evolved a symbiotic interrelationship with microorganisms which enables them to convert huge amounts of plant polymers (cellulose) that cannot be degraded by animal digestive processes.

Chapter 2 describes an 'artificial rumen' digester which allows the

maintenance of a mixed rumen microbial population under conditions similar to those in the rumen. In this acidogenic reactor high loads of grass (35 $g.l^{-1}.d^{-1}$) were digested at a solid retention time of only 60 h. The main fermentation products consist of volatile fatty acids (VFA), methane and carbon dioxide. In order to prevent an acidification of the reactor content, fluid containing the acid fermentation products was continuously removed through a filter unit and subsequently fed to a UASB-type methane reactor (Chapter 4). After conversion of the VFA the effluent of the methane reactor could be recirculated into the acidogenic reactor. Because the two-stage system is based on processes and microorganisms of the ruminant it was termed 'rumen-derived anaerobic digestion' (RUDAD) process. During the digestion of pure cellulose biogas production amounted to 0.7 $m^3.kg^{-1}$ of cellulose added, which is almost equal to the theoretical maximum value. Cellulose digestion was complete up to a loading rate of 25.8 g Volatile Solids (VS).$l^{-1}.d^{-1}$.

The role of rumen ciliates in the acidogenic phase digestion is discussed in Chapter 3.

Chapter 5 describes the results of the digestion of various solid organic waste materials in the acidogenic stage of the RUDAD-process. Degradation efficiencies were highest for waste materials with a low content of cell wall polymers (vegetable auction waste, horticultural waste, verge grass, organic fraction of municipal solid waste), but also for cellulosic (papermill sludge, refuse derived fuel) and even for lignocellulosic wastes (straw, bagasse, coffee pulp) high-rate conversions were obtained. In spite of a lignin content of 13%, cellulose degradation of bagasse amounted to 60-65% at a loading rate of 18$g.l^{-1}.d^{-1}$.

Optimal conditions of loading rate and retention of solids and liquids during the digestion of refuse derived fuel (RDF) were established at 23.4 g VS.$l^{-1}.d^{-1}$, 90 and 8-24 h, respectively (Chapter 6). The stability of the RUDAD-process was established in long-term experiments with RDF as a digester feed (Chapter 7). After prolonged recirculation of the fluid within the two-stage process, part of the VFA-catabolizing activities of the methanogenic phase had been taken over by the acidogenic phase (Chapters 4 and 7).

The economical feasibility of this novel rumen-derived process is determined not only by the rate of digestion but also by the simplicity of the reactor design. Chapter 8 describes the combination of rumen microorganisms and colonized polyurethane support particles carrying the VFA-catabolizing bacteria from the second phase in one reactor,resulting in a high-rate degradation of papermill sludge.

# SAMENVATTING

Jaarlijks wordt naar schatting 15 à $20.10^{10}$ ton plantaardig materiaal gevormd via het proces van de fotosynthese. De mineralisatie van deze plantaardige biomassa is vrijwel uitsluitend een microbiologisch proces dat zowel in aerobe als ook in anaerobe milieus verloopt. Tijdens de anaerobe mineralisatie wordt organisch materiaal omgezet in een mengsel van methaan en kooldioxide (biogas). Het proces komt vrijwel overal in de natuur voor, waar organisch materiaal wordt opgehoopt en de toevoer van zuurstof beperkend is, zoals in de sedimenten van rivieren en meren, moerassen, sawa's, maar ook in het spijsverteringsstelsel van herbivoren. Daarnaast vindt een gecontroleerde anaerobe afbraak plaats in door de mens geconstrueerde gistingsinstallaties. Methaangisting wordt reeds vanaf het eind van de vorige eeuw toegepast bij de stabilisatie van zuiveringsslib dat wordt gevormd tijdens de aerobe waterzuivering. De belangstelling voor anaerobe afvalwaterzuivering is de laatste jaren sterk toegenomen door de ontwikkeling van reactortypen waarin de aktieve biomassa is opgehoopt door middel van immobilisatie, zodat hogere belastingen kunnen worden bereikt. De anaerobe verwerking van vast organisch afval wordt echter nog relatief weinig toegepast, hetgeen vooral te wijten is aan de trage omzettingssnelheden en de instabiliteit van de gebruikelijke 1-traps reactoren (bijv. voor mest en rioolslib). De snelheidsbepalende stap in de afbraak van plantaardig afval wordt gevormd door de enzymatische hydrolyse van de celwandpolymeren (vnl. cellulose) tot oplosbare componenten. De snelheid waarmee de anaerobe afbraak verloopt zou aanzienlijk verbeterd kunnen worden door toepassing van micro-organismen met hoge cellulase aktiviteit in combinatie met een reactorontwerp dat voorziet in optimale milieucondities voor de betrokken organismen.

In dit proefschrift wordt een nieuw 2-traps vergistingsprocedé beschreven dat gebruik maakt van een unieke microbiële populatie

afkomstig uit de pens van herkauwers. De pens kan worden beschouwd als een van de meest efficiënte anaerobe ecosystemen voor wat betreft de microbiële afbraak van plantaardige biomassa. Herkauwers leven van plantaardig materiaal, maar beschikken niet zelf over de enzymen die verantwoordelijk zijn voor de hydrolyse van plantaardige celwandpolymeren. Hiervoor zijn ze volledig aangewezen op micro-organismen (vnl. ciliaten en bacteriën) die in grote aantallen in de pens voorkomen.

Door de procescondities nauwgezet na te bootsen bleek het mogelijk bacteriën en ciliaten afkomstig uit de pens van een schaap te handhaven in de eerste reactor van het 2-traps vergistingssysteem (hoofdstuk 2). In deze reactor werden hoge belastingen aan gras (35 $g.l^{-1}.dag^{-1}$) binnen een tijd van 60 uur voor zo'n 60 tot 70% afgebroken. De voornaamste eindproducten van het fermentatieproces in deze reactor zijn, evenals in de in vivo situatie, laagmoleculaire vluchtige vetzuren, methaan en kooldioxyde. Om verzuring te voorkomen, dient de vloeistof retentietijd in de eerste reactor (zuurvormende reactor) aanzienlijk korter te zijn dan de retentietijd van de vaste stof. De scheiding in de doorstroomsnelheden voor vaste stof en vloeistof bleek van essentieel belang voor het handhaven van grote aantallen ciliaten en een goede afbraak efficiëntie. De scheiding tussen vast en vloeibaar werd gerealiseerd door middel van filtratie. De in de vloeistof opgeloste vetzuren worden middels een in serie geschakelde methaanreactor van het type UASB omgezet in biogas. Nadat de vetzuren in de methaanreactor waren afgebroken, kon de vloeistof weer worden teruggeleid in de zuurvormende reactor (Hoofdstuk 4). Wanneer puur cellulose werd afgebroken in het 2-traps systeem, werd een gasproductie bereikt van 0.7 $m^3/kg$ cellulose (60% methaan), hetgeen vrijwel gelijk is aan de theoretisch maximaal haalbare waarde. De cellulose afbraak was volledig zelfs bij een volumebelasting van 25.8 g organische stof (VS).$l^{-1}.d^{-1}$.

De bijdrage van ciliaten in het afbraakproces in de zuurvormende reactor wordt besproken in hoofdstuk 3.

Diverse afvalmaterialen van verschillende herkomst en samenstelling bleken goed afbreekbaar in de zuurvormende reactor (Hoofdstuk 5). De afbraak verliep het meest efficiënt met afvalmaterialen met een laag gehalte aan celwandpolymeren zoals veilingafval, tuinderijafval,

bermmaaisel of de organische fractie van huisvuil. De afbraak van cellulose rijke (papierslib, RDF-huisvuil) en zelfs van lignine rijke afvalmaterialen (stro, bagasse, koffiepulp) verliepen echter ook efficiënt. Ondanks een lignine gehalte van 13% werd het cellulose in bagasse nog voor zo'n 60-65% afgebroken bij een belasting van 18 $g.l^{-1}.dag^{-1}$.

Voor de vergisting van de RDF-fractie uit huisvuil werden de optimum condities betreffende belasting, vaste stof en vloeistof retentietijd vastgesteld op respectievelijk 23.4 g $VS.l^{-1}.dag^{-1}$, 90 uur en 8-24 uur (Hoofdstuk 6). De stabiliteit van het 2-fasen systeem werd bepaald aan de hand van een langdurige fermentatieperiode, waarbij RDF als substraat werd toegediend (Hoofdstuk 7). Na langdurige recirculatie van de vloeistof binnen het 2-traps systeem, bleek een deel van de vetzuurafbrekende aktiviteit van de methaanreactor overgenomen te zijn door de verzuringsreactor hetgeen de stabiliteit bevordert.

Voor de economische haalbaarheid van het 2 traps procedé op industriële schaal zal naast de efficiëntie en het rendement van het vergistingsproces tevens de procestechnologische uitvoering van belang zijn. Door vetzuurafbrekende en methaanvormende bacteriën te immobiliseren op polyurethaan bleek het mogelijk de zuurvorming en methaanvorming gecombineerd in een reactor te laten plaatsvinden (Hoofdstuk 8). Dit lijdt tot vereenvoudigde procesvoering, hetgeen vooral van belang is voor de vergisting van afvalmaterialen die normaal filterverstopping zouden veroorzaken.

# DANKWOORD

Ofschoon op de omslag van dit boekje slechts 1 auteur wordt vermeld, moge het duidelijk zijn dat velen aan de totstandkoming ervan hebben bijgedragen.

Het leeuwendeel van de praktische werkzaamheden werd verricht door studenten biologie en scheikunde in de vorm van een bij- of hoofdvak microbiologie. Daarvoor mijn dank aan Anne ter Elst, Pieter van Gelder, Wim van der Laar, Piet Derikx, Ron de Ruyter, Marcel Teunissen, Eric Roersma, Maria Veldhuis, Peter Cox, Frans Vek, Erik Schellekens, Frank Verhagen, Theo Schoenmakers, Ward Hagemeyer, Gerard Verkley, Martin Gerhardus, en Karel Herberigs. Daarnaast dank ik Kor, Fried, Frederico, Huub op den Camp, Henk en Els voor de vele inspirerende discussies binnen de 'toegepaste groep'. Verder dank ik alle (ex-) collega's en studenten voor de prettige sfeer op 'onze' soms ietwat rommelige, maar zeer gezellige afdeling.

Zeer erkentelijk ben ik ook de medewerkers van de proefboerderij en de afdelingen glasblazerij, offset, illustratie en fotografie voor hun goede en snelle service.

Voor het aanleveren van de testmaterialen die in de diverse hoofdstukken aan de orde komen dank ik de papierindustrie (PAGE, Celtona, KNP, de Hoop), Rijkswaterstaat (bermmaaisel), Fa. Emons (champost), de VAM (huisvuilfracties ), Fa. Mol (uienafval), Haskoning (koffiepulp, bagasse), Spitman (tuinderijafval) en de AVEBE (aardappelafval).

Ik dank de leden van de STW-gebruikerscommissie Mw drs Oei (STW), Prof Dr Veldkamp (RU-Groningen), Ir Visser (VAM), Ir Kruijdenberg (PEO), Ing de Jong (PAGE), Dr van Voorneburg, Dr Doddema (TNO), Dr de Zeeuw, Dr Meiberg (AVEBE), Dr Ir Heijnen (Gist-Brocades), Dr A Cohen (CSM), Prof Dr Sassen, Prof Dr Ir Vogels, Dr Zwart, Dr Lubberding en Mr Aalbers (KU-Nijmegen), voor de stimulerende discussies.

Last, but certainly not least dank ik jou Inge voor de snelle en accurate verwerking van de tekst en voor de vele lay-out tips.

# CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 1 juni 1958 te Kerkrade. Het diploma VWO-B werd behaald in 1976 aan het Sancta Maria College in dezelfde plaats. In september van dat jaar begon hij met de studie biologie aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen werd in 1979 afgelegd. Zijn doctoraalfase was opgebouwd uit de bijvakken Chemische Cytologie (Dr. F. Wanka) en Cytologie en Histologie (Dr. W. Eling) en het hoofdvak Microbiologie (Prof.Dr.Ir. G.D. Vogels), en werd in 1982 afgesloten met het afleggen van het doctoraalexamen. Vanaf oktober 1982 tot oktober 1986 was hij werkzaam als wetenschappelijk medewerker op het Laboratorium voor Microbiologie van de Katholieke Universiteit te Nijmegen in het kader van een door de Stichting voor de Technische Wetenschappen (STW) gefinancierd onderzoek.

# STELLINGEN

behorende bij het proefschrift

## ANAEROBIC DIGESTION OF CELLULOSIC WASTE BY A RUMEN-DERIVED PROCESS

Nijmegen, 26 maart 1987. H.J. Gijzen

I

In studies waarin lignine wordt gebruikt als interne standaard ter bepaling van de afbraak van plantaardig materiaal door herkauwers, wordt geen rekening gehouden met het feit dat laagmoleculair lignine wordt opgelost tijdens de afbraak van celwandpolymeren.

II

Bij schattingen van de potentiële methanogene activiteit ($q_{CH_4(F_{420})}$) van anaeroob slib aan de hand van het coenzym $F_{420}$ gehalte, dient een onderscheid gemaakt te worden tussen de $F_{420}$ varianten in hydrogenotrofe ($F_{420}$-2) en acetoclastische ($F_{420}$-4 en $F_{420}$-5) methaanbacteriën.

*Dolfing J, Mulder JW (1985) Appl Environ Microbiol 49: 1142-1145.*
*Gorris L, van der Drift C (1986) In: Biology of anaerobic bacteria (Dubourguier et al, eds). Elsevier Sci Publ, Amsterdam, p144-150.*

III

De door Imai en Ogimoto beschreven bacteriën *Streptococcus bovis* en *Ruminococcus albus*, gehecht aan ciliaten uit de pens van een schaap, zijn waarschijnlijk methaanbacteriën.

*Imai S, Ogimoto K (1978) Jpn J Vet Sci 40: 9-19.*

IV

De toename van de malaria frequentie die wordt waargenomen bij zwangere vrouwen in holo-endemische malaria gebieden is een gevolg van de functionele interactie tussen het immuunsysteem en het endocriene stelsel.

*Vleugels MPH (1984) Proefschrift KU Nijmegen.*
*van Zon AAJC (1984) Proefschrift KU Nijmegen.*

V

De biotechnologische toepassingsmogelijkheden van anaerobe schimmels uit de pens zijn tot dusverre onderschat.

VI

In de literatuur vermelde methaanopbrengsten hoger dan 0.395 $m^3$ (35°C,1 atm) per kg chemisch zuurstofverbruik (CZV) zijn onjuist.

VII

Tijdens de bereiding van champignoncompost wordt slechts in een klein deel van de composthoop een temperatuur gehandhaafd die optimaal is voor microbiële activiteit.

VIII

Het voorstel van Malik en Tauro om ethaan en propaan in plaats van methaan te winnen uit anaerobe reactoren, lijkt gezien de argumenten die zij hiervoor aanvoeren meer op "science fiction" dan op "science".

*Malik RK, Tauro B (1986) TIBTECH 4: 305-307.*

IX

De "zure regen" in gebieden met een intensieve veeteelt is veelal een basisch neerslag.

X

Het feit dat het sterftecijfer ten gevolge van vele tropische ziektes, zoals malaria, veel hoger is dan dat van "westerse" ziektes, zoals kanker of hart- en vaatziekten, zou tot uitdrukking moeten komen in een andere verdeling van onderzoeksgelden op medisch gebied.